昭和32年7月9日 第三種郵便物認可
第45巻第6号 昭和33年6月25日発行
毎月1回25日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 6, June 25, 1958

Vol. 45, No. 6 **June, 1958**

THE JOURNAL
OF
BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI **SHIGERU AKAMATSU**
SHICHIRO AKIYA **FUJIO EGAMI**
SABURO FUNAHASHI **YOSOJI ITO**
KOOZOO KAZIRO **KATASHI MAKINO**
TOMOO MIWA **ZIRO NIKUNI**
NORIO SHIMAZONO **YUSUKE SUMIKI**
HIROSHI TAMIYA **ITARU WATANABE**
HARUHISA YOSHIKAWA

PUBLISHED MONTHLY
BY
THE JAPANESE BIOCHEMICAL SOCIETY
Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle	**Maruzen Co. Ltd.**	**Santo Vanasio**
Rutlant, Vt.	Tokyo	Köln
U.S.A.	Japan	Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Departments of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U. S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku Tokyo, Japan.

The Journal of Biochemistry, Vol. 45, No. 6, 1958

TURBIDOMETRIC TITRATION OF SERUM-PROTEIN

IV. TURBIDOMETRIC TITRATION OF SUBFRACTION OF BOVINE PLASMA ALBUMIN*

BY MASARU SOGAMI

(*From the Department of Physiology, Faculty of Medicine, Kyoto University, Kyoto*)

(Received for publication August 14, 1957)

So far, in sereral laboratories, by using various methods, such as chromatogrphic, (*1–4*), electrophoretic (*5–11*), immunological methods and metod using difference in solubility (*12*, *13*), human and bovine serum albumin were subfractionated into three or six components.

The author has investigated turbidometric titration (*14–17*) of protein solution and discovered that turbidometric diagram of serum albumin and γ-globulin had many peaks and that the author's method was capable of detecting a very subtle difference of serum albumin, such as species specificity (*17*). In this report, the author compared his results with chromatographic one under the following two headings: (I) turbidometric titration of chromatographic subfraction of bovine plasma albumin and (II) change in solubility of eluted bovine plasma albumin.

METHOD AND MATERIALS

By following the previous reports (*15*, *16*), turbidometric titration was carried out at 20° by using ammonium sulfate solution (saturated at 17°) as a precipitant and 0.11 *M* pH 6.80 or 0·4 *M* pH 6.80 Na-phosphate buffer as a solvent. While in the previous report, NaCl was dissolved into phosphate buffer, in this experiment NaCl was not dissolved into buffer. Protein solution was diluted to 0.02∼0.03 per cent with buffer solution, from which 8.0 ml. was used for turbidometric analysis.

Colnmn chromatography of bovine plasma albumin was carried out by using hydroxylapatite column (1.5 × 11 cm.) as adsorbent (*4*) and 0.02 *M*, 0.04 *M*, 0.07 *M*, 0,11 *M* and 0.4 *M* Na-phosphate buffer as an eluent. Elution rate was about 10∼15 ml. per hour and effluent was fractionated into 3.4 ml. of fraction by using a hand-made fraction collector. And also, chromatography was usually carried out at 10∼15°. Protein content in the different fractions was analysed by the extinction at 280 mμ in 1 cm. cell in a Beckman spectrophotometer.

Paperelectrophoretic analysis was made on 0.02∼0.03 ml. of 4 per cent bovine plasma albumin solution in veronal buffer (pH 8.5, $\mu=0.045$) according to Grassmann's method (*18–20*). Five 30 × 365 mm. strips (Toyo filter paper No. 131) were run simultaneously in one cell for 10 hours at 6°∼10° under constant current condition of 0.54 mA per 1 cm.

---

* These data were represented at General Meeting of physiological Society of Japan at Kobe Medical College in May, 1957.

These strips were stained by using brom phenol blue (*21*) and electrophoretic patterns were recorded by transmission densitometry. Experimental material used in these experiments was Armour's bovine plasma albumin. And also, paperelectrophoretic, turbidometric and chromatographic patterns were shown in Fig. 1.

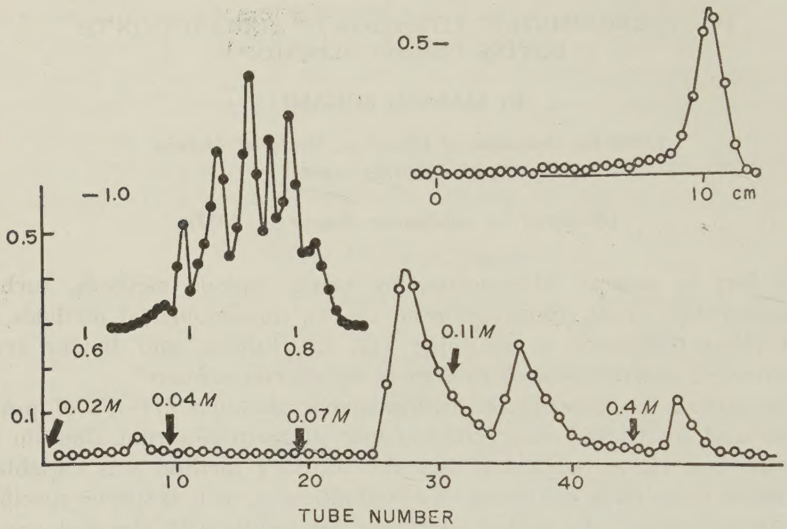


FIG. 1. The upper part shows paperelectrophoretic pattern of bovine plasma albumin. The abscissa and the ordinate show extinction and migration distance (cm.), respectively. The middle part shows turbidometrie diagram of bovine plasma albumin. The abscissa and the ordinate show volume fraction, C, of saturated ammonium sulfate solution and $\Delta T/\Delta C$, respectively, where T is relative amount of salted-out protein. The experimental conditions were as follows: protein concentration about 0.03 per cent, pH 5.64 at final point of experiment, pH 5.75 at 50 per cent saturation. The lower part shows chromatogram of bovine plasma albumin. The abscissa and the ordinate show tube number of fraction collector (one tube, 3.4 ml.) and extinction at 280 mμ, respectively. The elution was carried out at 19° by using the following buffer; 0.02 *M*, 0.04 *M*, 0.07 *M*, 0.11 *M* and 0.4 *M* pH 6.8 Na-phosphate buffer. See text for other experimental conditions.

EXPERIMENTAL RESULTS

Material used in these experiments was bovine plasma albumin (Armour's laboratory). While electrophoretic analysis of bovine plasma albumin in veronal buffer at pH 8.5 showed only one peak, chromatographic and turbidometric patterns showed several peaks.

Chromatographic analysis was caried out at 14° under conditions similar to Tiselius' report (*4*). Namely, bovine plasma albumin (17 mg.) was adsorbed at a buffer concentration of 0.02 *M* Na-phosphate pH 6.78, and elution was first carried out with this buffer and then with different buffer concentration as illustrated in lower part of Fig. 1. The first peak eluted with 0.07 *M* Na-phosphate buffer pH 6.81, the second with 0.11 *M*, pH 6.80 and the third with 0.4 *M*, pH 6.80. These results were not inconsistent with

Tiselius' report.

Turbidometric titration of 0.03 per cent bovine plasma albumin solution was carried out at 20° using 0.11 *M*, Na-phosphate buffer pH 6.80 as a solvent and ammonium sulfate solution (saturated at 17°) as a precipitant. Then, turbidometric diagram of bovine plasma albumin had six or seven peaks as shown in Fig. 1, middle part and in Fig. 3, upper part.

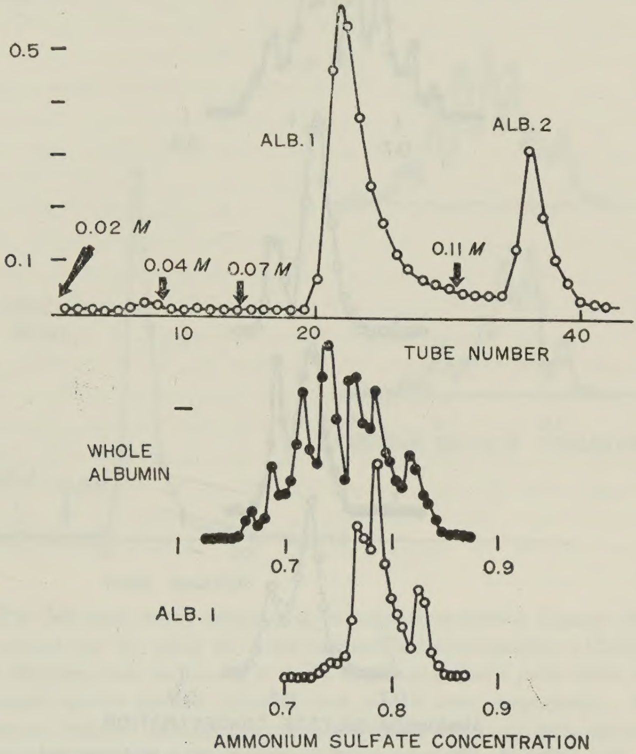


FIG. 2. The upper part shows chromatogram af bovine plasma albumin. The middle and lower parts show turbidometric diagrams of whole bovine plasma albumin and subfraction (albumin-I), respectively. See text and Fig. 1 for experimental conditions.

Fig. 2, upper part showed an experiment with 23 mg. of bovine plasma albumin on column (1.5×10 cm.). Since Tiselius *et al.* (*4*), reported that the first subfraction contained only albumin monomer and that the second and third contained albumin monomer and dimer, turbidometric analysis of the first was carried out. Turbidometric diagram of the first subfraction (0.026 per cent) had three peaks as shown in Fig. 2, lower part. As above stated, the turbidometric diagram of bovine plasma albumin had at least six peaks, but the diagram of the first subfraction had only three peaks. Therefore, the author did the same experiment three-times under the similar conditions but diagrams of the first subfraction were slightly different from those illustrated in Fig. 2. Namely, turbidometric diagram of the first subfraction had only two peaks as shown in Fig. 3, lower part. This

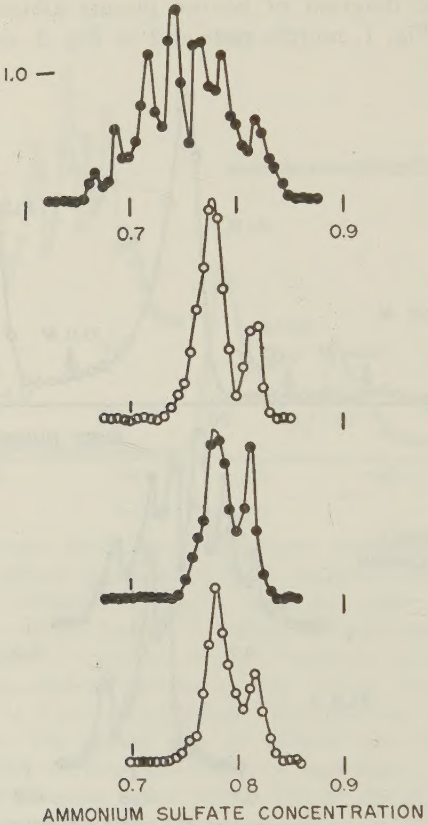


FIG. 3. The upper and lower parts show turbidometric diagram of whle bovine plasma albumin and subfraction (albumin-I), respectively. See text and Fig. 1 for experimental conditions.

difference may be due to slight alteration of eluted bovine plasma albumin. And also, though the first subfraction of column chromatography was about 50 per cent of the whole bovine plasma albumin, turbidometric diagram of the first subfraction did not consist with main peak in the diagram of the whole bovine plasma albumin. This difference also may be due to slight alteration of eluted bovine plasma albumin in the column.

The author carried out the following experiment. 36.4 mg. of bovine plasma albumin was adsorbed at a buffer concentration of 0.02 M, pH 6.78 Na-phosphate buffer, and elution was carried out with this buffer (94 ml.) at 4°. And then, elution was carried out at 16° with 0.04 M pH 6.77 and

0.4 M, pH 6.80 Na-phosphate buffer as shown in Fig. 4, left part. Turbidometric titration was carried out under the conditions similar to the above stated one, except for solvent (0.4 M, pH 0.80 Na-phosphate buffer). Turbidometric diagram of original bovine plasma albumin was shown in Fig. 4, right upper part and the one of eluted bovine plasma albumin was shown in Fig. 4, right lower part. There was a significant difference between two diagrams.

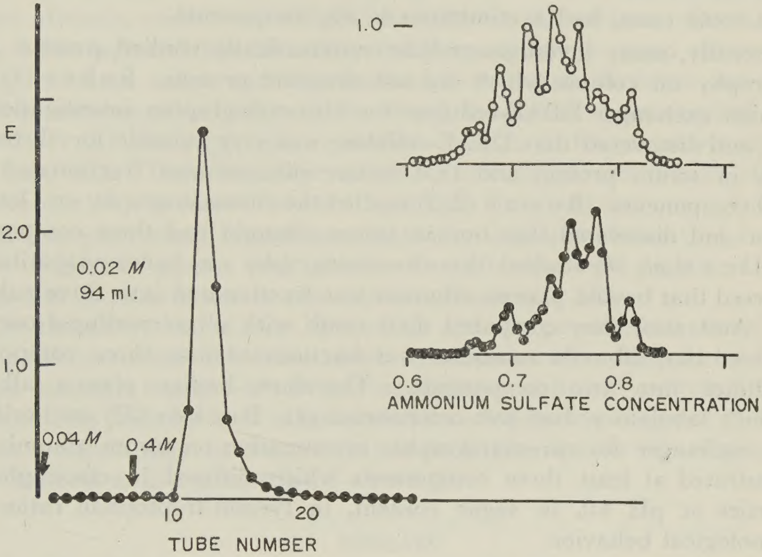


FIG. 4. The left part shows column chromatogram of bovine plasma albumin. The elution was carried out by using the following buffer concentration; 0.02 M, 0.04 M and 0.4 M pH 6.8 Na-phosphate buffer. The right upper and lower parts show turbidometric diagrams of whole bovine plasma albumin and eluted one, respectively. Experimental conditions were as follows; protein concentration 0.035∼0.03 per cent, solvent 0.4 M pH 6.79 Na-phosphate buffer, pH 5.94 at 50 per cent saturation and pH 5.85 at the final point of experiment.

DISCUSSION

(A) At lower pH region than isoelectric point of serum albumin, electrophoretic patterns of serum albumin showed the microheterogeneity. Luetscher (*5*, *6*) and Alberty (*9*) reported that bovine plasma albumin were resolved into three components and human serum albumin into only two components. Saifer *et al.* (*7*, *8*), reported that human and a bovine serum albumin were resolved into four components in acetate-NaCl buffer at pH 4.0, ionic strength 0.05 and that ratio of these components showed species specificity. And also, at higher pH region than isoelectric point of serum albumin, prolonged electrophoresis showed the microheterogeneity of serum albumin. Hoch *et al.* (*22*) reported that the prolonged electrophoresis of human serum albumin had two components in phosphate buffer at pH 8.0, ionic strength 0.1. And also, Raacke (*10*) reported that prolonged starch grain zone electrophoresis of bovine serum albumin showed two components in phosphate buffer at pH 7.0 and in cacodylate buffer at pH 6.5. Larson *et al.* (*11*) investigated the subfraction of human serum albumin using continuous filter paper electrophoresis in veronal buffer (pH 8.6, $\mu=0.05$) and immunological method (gel diffusion method). They discovered that human serum albumin had at least three components, and that in some cases, had a mimimum of six components.

Recently, many investigators have systematically studied protein chromatography on column which did not denature protein. Sober (*1*) used the anion exchanger DEAE-cellulose for chromatographic investigation on serum, and discovered that DEAE-cellulose was very suitable for chromatography of serum protein and that serum albumin was fractionated into several components. Boman (*2*, *3*) studied the chromatography on Dowex-2 column and discovered that human serum albumin had three components. Tiselius *et at*, (*4*) studied the chromatography on hydroxylapatite and discovered that bovine plasma albumin was fractionated into three subfractions. And also, they compared their result with ultracentrifugal one and discovered that albumin monomer was fractionated into three components and dimer into two components. Therefore, bovine plasma albumin (Armour's labolatory) had five components (*4*). Becker (*23*) used cellulose anion exchanger for chromatographic investigation on serum albumin and demonstrated at least three components which differed in electrophoretic properties at pH 4.0, in sugar content, in tyrosin-tryptophan ratios, and immunological behavior.

Method using difference in solubility has been investigated by Derrien's school (*12*, *13*) and the subfractionation of serum albumin was reported. Since 1954, the author used turbidometric titration method for the investigation on microheterogeneity of serum albumin, and discovered that human, goat serum albumin and bovine plasma albumin had several peaks in turbidometric diagram and that turbidometric diagram of serum albumin showed the species specificity (*16*). And also, the author discovered that turbidometric diagram of serum albumin changed according to the method in obtaining serum (*15*, *16*). On these experimental basis, the author recently concluded that each peak in turbidometric diagram might have one-to-one correspondence with real protein fraction (*15*).

(B) In this paper, in order to investigate the physicochemieal meaning of turbidometric diagram, the author studied albumin subfraction which was separated from bovine plasma albumin by using Tiselius' column chromatography (4). The author's chromatographic results were consistent with Tiselius' one as shown in Figs. 1 and 2. Though turbidometric diagram of bovine plasma albumin had at least six peaks, the diagram of the first subfraction (eluted at 0.07 *M*) had only two peaks as shown in Fig. 3. This fact makes it probable that protein which has only one peak in turbidometric diagram may be prepared in nearest future.

(C) And also, the first subfraction of column chromatography was about 50 per cent of the whole bovine plasma albumin. However, turbidometric diagram of the first subfraction did not consit with main peak in that of original bovine plasma albumin. This fact may be due to the slight alteration of protein in column. Therefore, the author made a comparison of the original bovine plasma albumin with eluted one. There was significant difference between them as shown in Fig. 4. This fact indicated that bovine plasma albumin underwent a slight alteration in the column. On the other hand, it was reported that the removal of fatty acid from serum albumin (by passage through anion exchange columns) increased dielectric increment, and that subsequent addition of Na oleate reduced it to the original value. Kendall (*25*) also reported a crystalline complex of human serum albumin with fatty acid. Therefore, slight alteration of eluted bovine plasma albumin may be due to the removl of small molecule from original one. And also, as previously reported, it may be due to the similar evidence that turbidometric diagrm of human and rabbit serum albumin were slightly alterated by the use of various serum separation methods (*15*, *16*).

On the above stated facts (B and C) and the previously reported facts, it is possible to say that each peak in turbidometric diagram of bovine plasma albumin has one-to-one correspondence with real protein fraction. And also, it is probable that many peaks in turbidometric diagram of bovine plasma albumin may be due to distinct molecular species or complex with small molecules, as reported by Sober (*1*).

SUMMARY

The author showed the turbidometric diagram of bovine plasma albumin and its subfraction, and slight alteration of eluted bovine plasma albumin. The conclusions were as follows:

1. Turbidometric diagrams of bovine plasma albumin and its first subfraction had seven and two peaks, respectively (See Fig. 3).
2. Turbidometric diagram of bovine plasma albumin was slightly alterated by passage through hydroxylapatite column.
3. Each peak in turbidometric diagram had one-to-one correspondence with real protein fractions. And also, these several components may be due to the distinct molecular species or the complex with small molecules.

The author wishes to thank Prof. K. Sasagawa (Dapartment of Physiology, Faculty of Medicine, Kyoto University) for his interest to my investigation. And also, the author wishes to thank to Dr. Y. Momotani (Department of Botany, Faculty of Science, Kyoto University) for his discussion.

REFERENCES

(*1*) Sober, H. A., Gutter, F. J., Wykoff, M. M., and Peterson, E. A., *J. Am. Chem. Soc.*, 78, 756 (1956)

Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1956)

(*2*) Boman, H. G., *Nature*, 175, 898 (1955)

(*3*) Boman, H. G., and Westland, L. E., *Arch. Biochem. Biophys.*, **64**, 217 (1956)

(*4*) Tiselius, A., Hjerten, S., and Levin, Ö., *Arch. Biochem. Biophys.*, **65**, 132 (1956)

(*5*) Luetscher, J. A., *J. Am. Chem. Soc.*, **51**, 2888 (1939)

(*6*) Luetscher, J. A., *J. Clin. Invest*, **19**, 313 (1940)

(*7*) Saifer, A., and Corey, H. J. *Proc. Soc. Exptl. Biol. Med.*, **87**, 203 (1954)

(*8*) Saifer, A., and Corey, H., *J. Biol. Chem.*, **217**, 23 (1955)

(*9*) Alberty, R. A., *J. Phys. Colloid Chem.*, **53**, 14 (1949)

(*10*) Reacke, I. D., *Arch. Biochem. Biophys.*, **62**, 184 (1956)

(*11*) Larson, D. L., and Feinberg, R., *Science*, **120**, 426 (1954)

(*12*) Derrien, Y., *Biochim et Biophys. Acta*, **8**, 631 (1952)

(*13*) Steyn-Parvé, E. P., *Biochim et Biophys. Acta*, **10**, 320 (1953)

(*14*) Sogami, M. Momotani, Y., and Inoue, Y., *Kagaku*, **10**, 851 (1955)

(*15*) Sogami, M., Momotani, M., and Inoue, Y., *J. Biochem.*, **44**, 137 (1957)

(*16*) Sogami, M., *J. Biochem.*, in press

(*17*) Sogami, M., *J. Biochem.*, 45, 349 (1958)

(*18*) Grassmann, W., and Hannig, K., *Hoppe-Seyler Z. physiol. Chem.*, **290**, **1** (1952)

(*19*) Valmet, E., and Svensson, H., *L K B Instrum. J.*, **1**, 3 (1954)

(*20*) Mori, G., and Kobayashi, M., Roshidenkieido no Jussai, Nankodo, Tokyo., 142 (1956)

(*21*) Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, **35**, 89 (1951)

(*22*) Hoch, H., and Morris, C. J. O. R., *Nature*, **156**, 234 (1945)

(*23*) Becker, E. L., in Sober, H. A., Gutter, F. J., Wykoff, M. M., and Peterson, E. A., *J. Am. Chem. Soc.*, **78**, 756 (1956)

(*24*) Hughes, W. L., The Proteins, Ed by H. Neurath and K. Baily, Acad. Press, New York, **2**, 683 (1954)

(*25*) Kendall, F. E., *J. Biol. Chem.*, **138**, 97 (1941)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

THE STRUCTURE OF CYTOCHROME C

III. THE GUANIDINATION OF CYTOCHROME C—BIOLOGICALLY ACTIVE DERIVATIVES OF CYTOCHROME C*

By KENJI TAKAHASHI, KOITI TITANI, KYOKO FURUNO, HISAYUKI ISHIKURA

(*From the Department of Chemistry, Faculty of Science, University of Tokyo, Tokyo*)

AND SHIGEKI MINAKAMI

(*From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo*)

(Received for publication, December 10, 1957)

In the previous paper (*1*) a description was made on the acetylation of cytochrome c. The acetyl-derivative was autoxidizable and could not accelerate oxygen uptake in succinic oxidase system if measured manometrically, although it was oxidized and reduced enzymatically. In the present article are described ths data concerning biologically active guanidino-derivative which is obtained by treating cytochrome c with *O*-methylisourea.

EXPERIMENTAL AND RESULTS

Cytochrome c—Cytochrome c was extracted from horse heart muscle and purified by the chromatographic method described in the previous paper (*1*).

O-Methylisourea—*O*-Methylisourea acid sulfate was prepared from *O*-methylisourea hydrochloride by the procedure of Hughes et al. (*2*). The crystallized material gave m.p. 106–112° (uncorrected). *O*-Methylisourea hydrochloride was synthesized from cyanamide, methanol and dry hydrochloride in this laboratory by the method of Stieglitz and McKee (*3*).

Preparation of Guanidino-Derivative of Cytochrome c—670 mg. of *O*-methylisourea acid sulfate (3.9 mM) was dissolved in 5 ml. of water at 0^0 and converted to the free base by addition of barium hydroxide. The mixture was centrifuged and the precipitate was washed several times with small amount of cold water. The washings were brought together with the supernatant fluid and adjusted to 25 ml. of pH 10.9–11.0. The solution was added to 100 mg. of lyophilized cytochrome c (7.1 μM). The reaction mixture was kept at 0^0 for 66 hours, and during this time aliquots were pippeted out at suitable intervals.

---

* A part of this study was presented at the "International Symposium on Enzyme Chemistry" Ocotober, 1957, Tokyo.

Each aliquot (2 ml.) was divided into four portions. The first portion (0.2 ml.) was diluted at once with 1/15 *M* phosphate buffer, pH 7.0 and used for the spectrophotometric study. The second portion (0.2 ml.) was dinitrophenylated with 2,4-dinitrobenzene fluoride for the determination of residual amino groups. The third portion (0.2 ml.) was hydrolyzed with 6 *N* HCl in a sealed tube at 120° for 24 hours for the determination of homoarginine residues. The fourth portion (1.4 ml.) was dialyzed succesively against runnig water for 3 hours and 1/15 *M* phosphate buffer for 2 days and used for the study of catalytic activity.

Estimation of the Number of Residual Amino Groups—Dinitrophenylated guanidino-derivative of cytochrome c was hydrolyzed with 6 *N* HCl in a sealed tudbe at 120° for 5 hours to solubilize the insoluble derivative and to destroy the absorption based on the heme part of cytochrome c. The hydrolyzate was extracted five times with ether and the aqueous layer was subjected to spectrophotometry at 400 mμ in acidic medium.

TABLE I

Guanidination of Cytochrome c

Sample No.	Reaction time (*hrs*)	Number of arginine and homoarginine residues	Number of ε-amino groups	Intensity of absorption at 550 mμ (%)	CO-bindidg affinity (%)	Activity (%)
0	0	3	18	100	20.2	100
1	0.5	4.3	16.7	91	17.7	105
2	1	5.2	15.5	82	19.7	105
3	2	7.0	14.7	87	18.3	109
4	3	7.6	13.6	91	19.3	100
5	5	8.9	12.1	88	18.6	105
6	11	10.1	9.6	90	26.1*	105
7	15	11.6	7.8	92	27.8*	95
8	20	12.1	6.7	92	28.2*	119
9	25	14.3	6.5	89	25.9*	114
10	36	16.3	2.9	88	28.0*	119
11	66	15.6	3.5	83	— **	114

* Small amount of precipitation was produced by bubbling carbon monoxide gas.

** By the same procedure, large amount of precipitation was produced and the estimation was impossible.

Estimation of the number of residual amino groups was made by assuming the reaction of *O*-methylisourea to be specific to ε-amino group in protein (*4*) and the number of ε-amino groups in horse cytochrome c to be 18 (*5*). The result is shown in Table I.

Estimation of the Number of Homoarginine Residues—The hydrolyzate of guanidinated cytochrome c was evaporated in a vacume desicator over sodium hydroxide and the residue was paper-electrophoresed using lutidine acetic acid buffer, pH 6.2 (*6*) to remove the unreacted *O*-methylisourea and its decomposition product by the acid hydrolysis which were found to inhibit the Sakaguchi reaction. The Sakaguchi reaction was applied to the fraction containing basic amino acids to estimate argninine and homoarginine together.

The estimation was made by assuming the number of arginine residues in the original horse hytochrome c to be 3 (*5*). The result is shown in Table I. The result agrees with that of DNP-method considerably well.

Spectrophotometric Study—The solution of guanidinated cytochrome c, which was diluted with phosphate buffer, pH 7.0, was reduced by addition of sodium dithionite and the intensity of the absorption at 550 mμ was calculated by assuming that of the starting solution to be 100. For the determination of the affinity toward carbon monoxide, pure carbon monoxice was passed for 5 minutes through the solution containing cytochrome c in the reduced state. The carbon monoxide-binding affinity was calculated according to the method of Tsou (*7*).

As shown in Table I, little change was observed in the intensity of the absorption at 550 mμ in the reduced state. In the estimation of the carbon monoxide-binding affinity, the change of stability as protein was observed in modified cytochrome c. That is, guanidino-derivative became to be precipitated by bubbling of carbon monoxide as the reaction proceeded. The estimation was thus impossible in the sample reacted fot 66 hours.

Measurement of Catalytic Activity—The electron transferring ability of guanidinated cytochrome c in succinic oxidase system was estimated manometrically using the enzyme system of rat liver as described in the previous paper (*1*). Enzymatic reducibility and oxidizability were separately examined by a qualitative method using a hand-spectroscope.

The result is shown in Table I. Guanidino-derivative of cytochrome c showed almost the same biological activity in this enzyme system as the original one. In the qualitative experiment, by which enzymic reduction and oxidation of cytochrome c were separately examined, no difference could be found between modified cytochrome c and the original one.

DISCUSSION

As indicated by the findings described above, it is sure that guanidine-derivative of cytochrome c, even when as much as 80 per cent of amino groups were guanidinated, has the same electron transferring ability in succinic oxidase system as the original one so far as the present estimation method is concerned. Considering the result of acetylation described in the previous paper (*1*), this findings seems to demonstrate the importance of cationic groups in the protein moiety for the function of cytochrome c. That is, cationic groups are concerned in making up the stereochemical structure specific to cytochrome c and when these are blocked with other non-cationic groups, for example, acetyl group, the specific structure is broken, yet when converted to other cationic groups, for example, guanidino group, such destruction is not caused. This supposition would be supported by the fact that acetylated cytochrome c is autoxidizable, while the guanidinated one is not.

Other properties, namely the absorption spectrum and the inertness towards carbon monoxide also showed no change in the present experiments. The only change observed in the present investigation was that of stability as protein of cytochrome c. Considering that it is well known for cytochrome c to be stable within a wide range of pH and temperature, such change seems to be probably due to the slight change in bulk of molecule caused by that of side chains of this enzyme.

SUMMARY

1. Cytochrome c was guanidinated with *O*-methylisourea and the number of amino groups reacted in the protein moiety was estimated by the DNP-method of Sanger as well as by the method using the Sakaguchi reaction.

2. Guanidino-derivative of cytochrome c showed the same biological activity in succinic oxidase system, the same absorption spectrum in the reduced state and the same non-reactivity towards carbon monoxide as the original one.

3. The only change observed in the present investigation was the decrease in stability of cytochrome c.

The authors wish to express their thanks to Prof. S. Akabori and Prof. H. Yoshikawa for constant guidances in the course of the work. Thanks are also due to Prof. Y. Yoneyama and Dr. M. Ishimoto for valuable discussions.

REFERENCES

(*1*) Minakami, S., Titani, K., Ishikura, H., *J. Biochem.*, **45**, 341 (1958)

(*2*) Hughes., W. L., Jr. Saroff, H. A., and Carney, A. L., *J. Am. Chem. Soc.*, **71**, 2476 (1949)

(*3*) Stieglitz, J., and McKee, R. H., *Ber. Chem. Ges.*, **33**, 1517 (1900)

(*4*) Greenstein, J. P., *J. Biol. Chem.*, **109**, 529, 641 (1935)

(*5*) Titani, K., Minakami, S., and Ishikura, H., reported in a Symposium on Protein Structure (at Fukuoka, Oct. in 1956).

(*6*) Titani, K., Ishikura, H., and Minakami, S., *J. Biochem.* **44**, 499 (1957)

(*7*) Tsou, C. L., *Biochem. J.*, **49**, 362, 367 (1951)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

ENZYMATIC PREPARATION OF OPTICALLY ACTIVE ESSENTIAL AMINO ACIDS

IV. THE PREPARATION OF L-LEUCINE AND L-VALINE A COMMENT ON THE TRANSAMINASE SYSTEMS OF HEART MUSCLE

By SETSUJI SAKURAI

(*From the Laboratory of Biochemistry, Faculty of Science, Osaka University, Osaka*)

(Received for publication, October 25, 1957)

In the previous paper (*1*) of this series, a method for the preparation of L-methionine by enzymatic transamination was described. This method involved two steps, namely, the preparation of α-keto-γ-methylmercaptobutyric acid by the hydrolysis of methyl α-ethoxalyl-β-methylmercaptopropionate, and the preparation of L-methionine from α-keto-γ-methylmercaptobutyric acid by enzymatic transamination.

In an attempt to prepare L-leucine by the same principle, it was found that the yield in the condensation reaction of ethyl *iso*-valerate with diethyl oxalate and the subsequent hydrolysis of the condensation product, ethyl α-ethoxalyl-*iso*-valerate, was relatively low. On the other hand, α-keto-*iso*-caproic acid was obtained by hydrolysis of *iso*-valeryl cyanide with concentrated hydrochloric acid in fairly good yield, although Tschelinzeff (*2*) reported that the preparation of α-keto-*iso*-caproic acid was not successful.

Alpha-keto-*iso*-valeric acid was obtained in the same way from isobutyryl cyanide. It was observed, however, that the yield of L-valine from α-keto-*iso*-valeric acid was lower than that of L-leucine from α-keto-*iso*-caproic acid due to instability of α-keto-*iso*-valeric acid and difficulty in separating L-valine from the reaction mixture.

This paper describes the procedures for the preparation of L-leucine and L-valine from the corresponding keto acids by heart muscle transaminase and some interesting observations on the enzymatic system of heart muscle.

EXPERIMENTAL AND RESULTS

Preparation of L-*Leucine*

Preparation of iso-Valeryl Bromide—Into a mixture of 30.6 g. (0.3 mole) of *iso*-valeric acid and 3.1 g. (0.1 gram atom) of dry red phosphorous was dropped 40.0 g. (0.25 mole) of dry bromine at 30° with vigorous stirring. When all the bromine was added, the flask was equipped with a condenser set for downward distillation. The isovaleryl bromide distilling at 134–135° weighed 43.6 g. (90 per cent of the calculated amount).

Preparation of iso-Valeryl Cyanide—In a 100 ml. flask fitted with a dropping funnel and a reflux condenser protected from the moisture by a calcium chloride tube was placed 18 g. of cuprous cyanide, and 20.3 g. of *iso*-valeryl bromide was dropped slowly from the funnel with swirling of the apparatus by hand so that all of the cuprous cyanide was moistened with the bromide. The mixture was then heated in an oil bath kept at 100–105°.

After an hour, the flask was equipped with a condenser set for downward distillation. Thirteen grams of crude *iso*-valeryl cyanide was collected in a receiver kept at −30° under diminished pressure of about 40 mm. Hg. The crude product was purified by fractional distillation. The *iso*-valeryl cyanide distilling at 126.5–128° weighed 12.3 g. (80 per cent of the calculated amount).

Preparation of α-Keto-iso-caproic Acid—In a 100 ml. long-necked flask equipped with a thermometer were placed 12 g. of *iso*-valeryl cyanide and 25 ml. of concentrated hydrochloric acid. The flask was shaken in a water bath kept at 50°. As this reaction was exothermic, cooling was applied to keep the temperature below 50°. After standing for one hour, the reaction mixture was cooled. Ammonium chloride formed by hydrolysis was removed by suction and 25 ml. of water was added to the filtrate. Tne upper layer was separated, the aqueous layer was extracted twice with 20 ml. of ether and the ether solution was combined with the upper layer. On the distilling off the ether *in vacuo*, there was obtained 12 g. of crude α-keto-*iso*-caproic acid.

The 2,4-dinitrophenylhydrazone of α-keto-*iso*-caproic acid was obtained as follows. The crude α-keto-*iso*-caproic acid (0.2 g.) was added to 2,4-dinitrophenylhydrazine solution (0.2 g. of 2,4-dinitrophenylhydrazine in 100 ml. of 2 N hydrochloric acid) and the reaction mixture was allowed to stand at 37° overnight. The crude hydrazone (0.2 g.) was reprecipitated from the alkaline solution with dilute hydrochloric acid and then recrystallized from 60 ml. of 30 per cent ethanol. The yield was 0.16 g. with m.p. 140–142°.

Analysis.	Calcd. for $C_{12}H_{14}N_4O_6$;	C, 46.45; H, 4.55; N, 18.06
	Found;	C, 46.50; H, 4.13; N, 17.94

Preparation of L-*Leucine*—A mixture of 11.8 g. of the crude α-keto-*iso*-caproic acid prepared as described above and 18.0 g. of L-glutamic acid was added to 300 ml. of water and the solution was adjusted to pH 7.6 with aqueous ammonia. To this solution was added 80 ml. of transaminase solution prepared from 60 g. of pig heart muscle and the total volume was adjusted to 300 ml. with water. The reaction mixture was placed in a 600 ml. Erlenmeyer flask at 38°. After 84 hours, the mixture was heated to 80° for 10 minutes and and the insoluble matter was filtered off. The clear filtrate was evaporated *in vacuo* to about 150 ml. and then the pH was adjusted to 3.2 with concentrated hydrochloric acid to precipitate the unreacted glutamic acid. After removing the precipitate, the filtrate was adjusted to pH 1.0 with dilute hydrochloric acid and then evaporated *in vacuo* to dryness. The residue was washed twice with 40 ml. of ether and then extracted with 40 ml. of methanol. After removing the methanol, the residue was dissolved in 150 ml. of water and decolorized with active carbon. The solution was passed through a column of Amberlite IR-4B (2×50 cm.), and then the solution was followed by 200 ml. of water. This treatment was repeated again. The effluent was concentrated in vacuo to dryness and the residue was washed with 10 ml. of 80 per cent methanol.

The residue, crude L-leucine, weighed 2.5 g. with decomposition point 240–250°. On recrystallization from 60 ml. of 50 per cent methanol, 0.8 g. of L-leucine with decomposition point 280–285° was obtained.

Analysis. Calcd. for $C_6H_{13}NO_2$;	C, 54.94; H, 9.99; N, 10.68
Found;	C, 54.78; H, 9.59; N, 10.52

$[\alpha]_D^{25°}$, 15.0° (C=0.03, 6 N HCl). According to Thomas (*3*) that of L-leucine is 14.85° (4 per cent in 6.02 N HCl, 25°).

Preparation of L-*Valine*

Preparation of iso-Butyryl Bromide—From 51 g. of isobutyric acid, 5 g. of red phosphorous and 76 g. of dry bromine, 81 g. (94 per cent of the calculated amount) of *iso*-butyryl bromide distilling at 107–113° was obtained.

Preparation of iso-Butyryl Cyanide—Starting from 50 g. of *iso*-butyryl bromide and 40 g. of cuprous cyanide, the same procedure as in the preparation of *iso*-valeryl cyanide gave 28 g. (85 per cent of the calculated amount) of *iso*-butyryl cyanide distilling at 107–115°.

Preparation of α-Keto-iso-valeric Acid—In a 200 ml. flask were placed 18 g. of *iso*-butyryl cyanide and 54 ml. of concentrated hydrochloric acid and the flask was shaken in a water bath kept at 50° until the exothemic reaction started. As this reaction proceeds more vigorous than hydrolysis of *iso*-valeryl cyanide, efficient cooling was necessary to keep the temperature at 50°. After keeping the mixture at 40° for an hour, the mixture was cooled and ammonium chloride was removed by suction. The filtrate was diluted with 100 ml. of water and extracted twice with 50 ml. of ether. The ether solution was washed with a small amount of water and ether was distilled off *in vacuo*. The residue weighed 15 g. Determination by column chromatography of 2,4-dinitrophenylhydrazone showed that the residue contained 6.1 g. of α-keto-*iso*-valeric acid.

The 2,4-dinitrophenylhydrazone of α-keto-*iso*-valeric acid was prepared as follows. The crude α-keto-*iso*-valeric acid (0.25 g.) was added to 2,4-dinitrophenylhydrazine solution (0.2 g. of 2,4-dinitrophenylhydrazine in 100 ml. of 2 N hydrochloric acid) and the reaction mixture was allowed to stand at 37° overnight. The hydrazone (0.2 g.) separated by suction was reprecipitated from the alkaline solution with dilute hydrochloric acid and then recrystallized from 50 per cent ethanol. The yield was 0.1 g. with m.p. 188°.

Analysis. Calcd. for $C_{11}H_{12}N_4O_6$;	C, 44.60; H, 4.08; N, 18.91
Found;	C, 44.14; H, 3.85; N, 18.62

Preparation of L-*Valine*—A mixture of 14.5 g. of the crude α-keto-*iso*-valeric acid prepared as described above and 20 g. of monosodium glutamate was dissolved in 200 ml. of water and the solution was adjusted to pH 7.6 with aqueous ammonia. To this solution was added 300 ml. of transaminase solution prepared from 100 g. of heart muscle and the mixture was kept at 38° for 72 hours. The reaction mixture was heated at 80° for 10 minutes and the insoluble matter was filtered off. The filtrate was evaporated *in vacuo* to about 60 ml. and then the unreacted glutamic acid was removed. The filtrate was adjusted to pH 1.0 with hydrochloric acid and evaporated *in vacuo* to dryness. The residue (23 g.) was extracted with 80 ml. of absolute methanol and the methanol solution was dried *in vacuo.* The residue (13 g.) was dissolved in 30 ml. of water and free hydrochloric acid was removed in vacuo. The residue was dissolved in 150 ml. of water and the solution was passed through a column of Amberlite 1R–4B (2×50 cm.) and the solution was followed by 200 ml. of water. The effluent was dried in vacuo and the residue (9 g.) was dissolved in 60 ml. of water. To this solution was added 5 g. of copper carbonate and the mixture was heated at 60° for 30 minutes. After cooling, the insoluble matter was filtered off and the filtrate was dried *in vacuo.* The residue (3.8 g.) was extracted twice with 50 ml. of methanol and the methanol solution was dried *in vacuo.* The residue was dissolved in 40 ml. of water and treated with hydrogen sulphide and then filtered. The filtrate was evaporated *in vacuo* to dryness. The residue (1.5 g.), which decomposes at 250–260° was recrystallized from 6 ml. of 50 per cent ethanol giving 0.8 g. of crystals.

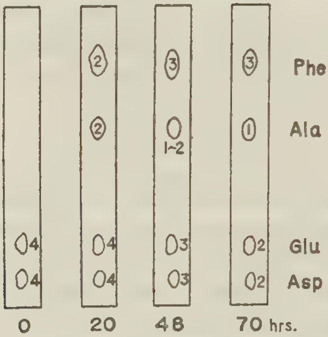


FIG. 1. Formation of L-alanine in the phenylpyruvate-aspartate-glutamate system.

The reaction mixture contained 0.61 mM of *p*-phenylpyruvic acid, 0.61 mM of L-aspartic acid, 0.2 mM of L-glutamic acid, 3.5 ml. of enzyme solution prepared from 1.65 g. of pig heart muscle and 3 ml. of 0.1 *M* phosphate buffer (PH 7.4). Total volume, 10 ml.; Temp., 37°. Samples were taken at the time specified, and deprotenized. The supernatant was examined by paper chromatography in the phenol-water (77 : 23 by volume). Numbers marked on spots indicate colour strengto of spot on an arbitrary scale, varying from 1 (just visible) to 4 (maximum intensity).

Decomposition point, 285–290°; $[\alpha]_D^{15°}$, 28.2° (C=0.016, 6 *N* HCl). According to Fischer (*4*) that of L-valine is 28.8° (3.4 per cent in 6.0 *N* HCl, 20°).

Some Observations in the Enzymatic Reaction

Formation of L-*Alanine in the Course of Aspartate-Glutamate-Phenylalanine Transamination*—Fig. 1 shows the formation of L-alanine concomitant with that of L-phenylalanine when L-aspartic acid (equimolar to phenylpyruvic acid) and a small amount of L-glutamic acid are used as amino donor. After 20 hours, the formation of L-phenylalanine was parallelled with that of L-alanine and after 48 hours, L-alanine tended to decrease whereas the formation of L-phenylalanine proceeded linearly and finally, after 70 hours, only a small amount of L-alanine was found.

TABLE. I

Formation of Amino Acid in Control Experiment

		α-Keto glutaric		α-Keto-γ-methylmercaptobutyric acid		Pyruvic acid	Phenyl-pyruvic acid	α-Keto-*isc*-valeric acid
			NH^+_4 was added[1]		NH^+_4 was added			
Keto acid added (μM)		290	290	260	260	210	300	230
” disappeared (μM)		29	22	36	33	210	—	58
Amino acid detected (μM)	Glutamic acid	7	8	3	4.5	4	—	—
	Methionine	0	0	18	35[2]	0	0	0
	Alanine	0	0	0	0	0	0	0
	Phenyl-alanine	0	0	0	0	0	30	0
	Valine	0	0	0	0	0	0	35

The reaction mixture contained neutralized solution of keto acid, 1.7 ml. of enzyme solution prepared from 0.8 g. of pig heart muscle and 2 ml. of 0.1 *M* phosphate buffer (pH 7.5). The total volume was adjusted to 5 ml. and kept at 37° for hours.

1) 0.27 ml. of ammonium chloride solution (250 mg/5 ml, pH was adjusted at 7.6 with aqueous ammonia solution) was added.

2) In addition to 35 μM of methionine, 1.5 μM of methionine sulfoxide was formed.

Although the experiments with other keto acids were not made, the same phenomenon will be observed when L-aspartic acid is used as main amino donor.

Formation of Amino Acid in Control Experiment—As shown in Table I, a considerable amount of amino acid was formed when phenylpyruvic acid, α-keto-γ-methylmercaptobutyric acid or α-keto-*iso*-valeric acid was incubated with enzyme solution alone. Although the enzyme solution contains free amino acids, it is difficult to consider that only these amino acids are responsible for the formation of respective amino acid in control experiment.

The amount of free amino acids, whic can easily be transaminated, does not exceed 6–12 μM in the experimental condition in Table I, if it is assumed not to be far different from the amount of free amino acids in liver tissue which has been determined by Moore *et al.* (*5*).

An interesting phenomenon was observed when α-keto-γ-methylmercaptobutyric acid was incubated with enzyme solution containing an equimolar amount of ammonium ion; as shown in Table I, a small amount of methionine sulfoxide was detected when α-keto-γ-methylmercaptobutyric acid was incubated with enzyme solution in the presence of ammonium ion, whereas in the absence of ammonium ion, methionine sulfoxide could not be detected and furthermore the formation of methionine was only half as much as that in the presence of ammonium ion.

DISCUSSION

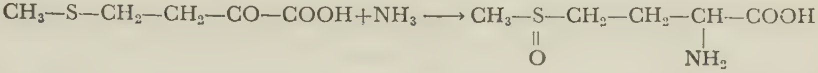
Fate of L-alanine in the course of preparation of L-phenylalanine in the presence of L-aspartic acid used as main amino donor does not seem compatible with the data in Table I, in which rapid disappearance of pyruvic acid without concomitant formation of L-alanine can be seen when pyruvic acid was incubated with enzyme solution.

It can be explained as follows; 1) A part of pyruvic acid formed from oxalacetic acid was converted to L-alanine by glutamic acid-alanine transaminase. 2) Alpha-ketoglutaric acid formed from L-glutamic acid proceeds to aspartic acid-glutamic acid transamination and has little chance to react with L-alanine in the initial stage and consequently L-alanine accumulated gradually. 3) When the amount of L-aspartic acid decreases (or the rate of inactivation of glutamic acid-aspartic acid transaminase exceeds that of glutamic acid-alanine transaminase), L-alanine tends to react with α-ketoglutaric acid and this equilibrium proceeds to the formation of pyruvic acid and glutamic acid. Consequently, in the final stage, only a small amount of L-alanine can be found in the reaction mixture.

The results shown in Table I, in which formation of L-phenylalanine, L-valine or L-methionine was shown when the corresponding keto acid was incubated with enzyme solution alone, suggested that some other processes than transamination, in which free amino acids in enzyme solution exert as amino donor, might be involved in these enzymatic reactions.

The effect of ammonium ion on the formation of methionine and methionine sulfoxide suggested that reductive amination might occur in this enzymatic reaction.

Since α-keto-γ-methylmercaptobutyric acid is susceptible to oxidizing reagent, it seems likely that the following reaction takes place enzymatically, methionine sulfoxide thus formed being reduced to methionine.



$$CH_3{-}S{-}CH_2{-}CH_2{-}CO{-}COOH+NH_3 \longrightarrow CH_3{-}\underset{\underset{O}{\|}}{S}{-}CH_2{-}CH_2{-}\underset{\underset{NH_2}{|}}{CH}{-}COOH$$

The experimental data for this assumption will shortly be published elsewhere.

SUMMARY

1. Alpha-keto-*iso*-caproic acid and α-keto-*iso*-valeric acid were prepared by hydrolysis of their corresponding acyl cyanides with concentrated hydrochloric acid.

2. L-Leucine was obtained from α-keto-*iso*-caproic acid by heart muscle transaminase. The yield of L-leucine from *iso*-valeric acid was 17 per cent, whereas that of L-valine from *iso*-butyric acid was only 6 per cent.

3. Fate of L-alanine formed in enzymatic transamination where L-aspartic acid is used as main amino donor was described.

4. A new reaction of methionine formation in biological systems was suggested.

The author wishes to thank Prof. S. Akabori for his kind guidance and also to Messrs. S. Yamada and T. Ito for their technical assistance.

REFERENCES

(*1*) Yamada, S., and Sakurai, S., *J. Biochem.*, **44**, 557 (1957)
(*2*) Tschelinzeff, W., and Schmidt, W., *Ber.*, **62**, 2210 (1920)
(*3*) Thomas, D. W., and Nieman, C., *J. Biol. Chem.*, **175**, 241 (1948)
(*4*) Fischer, E., and Jacobs, W. A., *Ber.*, **39**, 2942 (1906)
(*5*) Tallman, H. H., Moore, S., and Siein, W. H., *J. Biol. Chem.*, **211**, 932 (1954)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

COLORIMETRIC DETERMINATION OF UREA WITH SPECIAL REFERENCE TO THE AGINASE DETERMINATION

BY YUKIAKI NADAI

(*From the Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Kyoto*)

(Received for publication, October 2, 1957)

A number of methods for the determination of arginase activity have been reported. While the urease method by Hunter and his co-workers (*1*, *2*) had been used for about these decades, in recent years various kinds of methods involving direct determination of urea have been become more widely employed becuase of their practical convenience. Among them, the most popular are the α-isonitrosopropiophenone method developed first by Van Slyke and Archibald (*3*) and the xanthydrol method described by Everett and Phyllis (*4*). These methods, however, are not suitable for radid determination and have such disadventages that the reagents are too expensive for us at present.

More recently, Watt and Chrip (*5*) published a new spectrophotometric method for determination of urea, which is based upon a yellowish-green color produced when *p*-dimethylaminobenzaldehyde is added to the dilute hydrochloric acid solution of urea. The method was reported to be convenient for the practical use as well as its' high accuracy. The author modified this method to make it more suitable for the determination of arginase activity of tissues than the original report.

METHODS

p-Dimethylaminobenzaldehyde Reagent—A yellowish commercial preparation of this chemical was recrystallized three times according to the Adams and Coleman's method (*6*). The pure substance thus obtained is a colorless small plate crystall (m.p. 74°). The reagent was prepared by dissolving 2.0 g. of *p*-dimethylaminobenzaldehyde in 100 ml. of 95 per cent ethanol and adding 15 ml. of concentrated hydrochloric acid to it. It is very stable for over a few months when it is stored in a refrigerator.

Enzyme Solution—About one g. each of liver and kidney of a mouse, weighing from 15 to 20 g., was homogenized in a ground glass homogenizer with 10 times of volume of ice-cold distilled water, and then centrifuged at 3000 r.p.m. for 10 minutes. The supernatant, after it was properly diluted with water, if necessary, was used as the enzyme solution.

Incubation Mixture—A mixture of 1.0 ml. of the enzyme solution, 1.0 ml. of 0.05 M L-arginine solution of pH 6.0, 1.0 ml of 0.1 M veronal buffer of pH 9.2, and 1.0 ml. of 0.01 M $MnSO_4$ solution was incubated under the layer of toluene at 37°. At the end of a certain incubation period, 6.0 ml. of 10 per cent trichloroacetic acid was added to the mixture and after standing for 10 minutes the precipitates were filtered off. 5 ml. of the filtrate was used for the determination of urea by the photometric method.

Photometric Measurement of Urea Formed—To 5 ml. of the sample to be tested was added 2 ml. of *p*-dimethylaminobenzaldehyde reagent. A yellowish-green color developed immediately. After standing for 15 minutes at room temperature the extinction of the solution in a 20-mm. cuvette was read in a photoelectric colorimeter of Shimadzu Model U II, using a 450-mμ. filter.

RESULT AND DISCUSSION

As shown in Fig. 1, a standard curve, extinction *vs.* urea concentration, was found to be completely linear within the range between 0.15 mg. and 1.6 mg. of urea per 5 ml. of the sample. The effect of various compounds on the color development of the *p*-dimethylaminobenzaldehyde method was listed in Table I. It is ascertained from the table that veronal-Na, $MnSO_4$, and NH_4Cl have almost no effect, while arginine, ornithine, and trichloroacetic acid seem to have some effects on the color development and its development depends upon the experimetal condition. Tissue extract, on the other eand, seems to lessen the coloration by urea, as shown in Table II. However, these positive and negative effects of the addition of substances on the color development can be readily counterbalanced by the use of those standard curves which have been plotted in a set of tests with all the necessary components of the mixture for the arginase incubation arranged in such a way as that shown in Table III. The linearity of such standard curves is also obvious in Fig. 1.

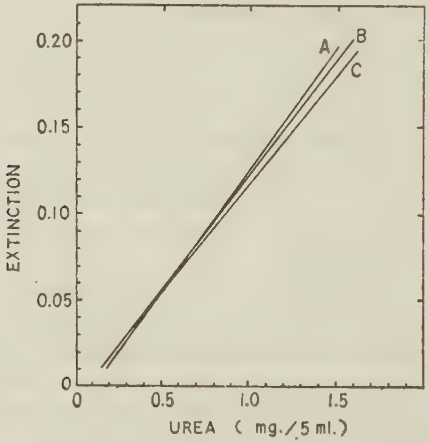


FIG. 1. Calibration curve for urea with *p*-dimethylaminobenzaldehyde at 450 mμ. Curve A, with no addition; curves B and C, with additions as listed in Table III (B 1 : 50 and C 1 : 10 homogenates).

TABLE I

Effect of Substance Added on the Color Development of Urea with p-Dimethylaminobenzaldehyde

To 1.0 ml. of water or 0.02 *M* urea solution were added appropriate volume of a substance to be tested and water to make up to a total volume of 5.0 ml. Colorimetric measurement was performed 15 min. after the addition of 2.0 ml. of the aldehyde reagent.

Substance added		Mixture without urea (control)	Mixture with 1.20 mg. of urea	
Compound	Final concent ration (*M*)	Extinction	Extinction	Urea (*mg.*)
Veronal-Na	0.001	0.000	0.143	1.20
	0.005	0.000	0.155	1.21
	0.001	0.005	0.156	1.22
	0.1	0.009	0.158	1.24
$MnSO_4$	0.001	0.000	0.153	1.10
	0.005	0.000	0.153	1.20
	0.01	0.007	0.155	1.21
	0.03	0.010	0.157	1.23
NH_4Cl	0.001	0.000	0.153	1.20
	0.005	0.000	0.153	1.20
	0.01	0.002	0.153	1.20
	0.03	0.004	0.157	1.23
L-Arginine	0.001	0.000	0.157	1.23
	0.005	0.005	0.160	1.25
	0.01	0.010	0.165	1.29
	0.02	0.017	0.175	1.35
L-Ornithine	0.001	0.000	0.158	1.24
	0.005	0.011	0.167	1.30
	0.01	0.015	0.178	1.38
	0.03	0.022	0.178	1.38
CCl_3COOH	2*	0.000	0.153	1.20
	4	0.006	0.156	1.22
	5	0.013	0.165	1.28

* Final concentration in per cent.

TABLE II

Effect of Mouse Liver Homogenates on the Color Development of Urea with p-Dimethylaminobenzaldehyde

To a mixture of 4.0 ml. of 10 per cent CCl_3COOH and 2.0 ml. of 0.01 *M* urea solution or water were added various volumes of the liver homogenates (1 :10 and 1 : 50) and water to make up to a total volume of 10.0 ml. The mixture was filtered and to 5.0 ml. of the filtrate was added 2.0 ml. of the aldehyde reagent.

Homogenate		Homogenate-mixture without urea	Homogenatemixture with urea	
Concentration	Addition (*ml.*)	Extinction	Extinction	Urea* (*mg.*)
1 : 10	0.1	0.005	0.069	0.60
	0.5	0.008	0.071	0.62
	1.0	0.022	0.074	0.64
1 : 50	0.1	0.002	0.070	0.60
	0.5	0.008	0.070	0.60
	1.0	0.010	0.072	0.62

* 100 per cent recovery ; 0.60 mg.

TABLE III

Composition of the Test Mixture for the Curves B and C in Fig. 1

All the substances added are listed in the Table. The mixture was centrifuged and to 5.0 ml. of the supernatant was added 2.0 ml. of the aldehyde reagent.

Substance added \ No. of test tubes	Volume of added solution (*ml.*)					
	1	2	3	4	5	6
10 per cent CCl_3COOH	4.0	4.0	4.0	4.0	4.0	4.0
Mouse liver homogenate (1 : 10 or 1 : 50)	1.0	1.0	1.0	1.0	1.0	1.0
0.1 *M* Veronal buffer	1.0	1.0	1.0	1.0	1.0	1.0
Urea (1.20 *mg./ml.*)	0	0.5	1.0	1.5	2.0	2.5
0.015 *M* L-Arginine	3.0	2.0	1.0	0.5	0.25	0
H_2O	1.0	1.5	2.0	2.0	1.75	1.5

SUMMARY

1. A simple and rapid colorimetric method is presented for the assay of the arginase activity of the mouse liver and kidney homogenate.

2. The incubation mixture for arginase action is deproteinized with trichloroacetic acid and the filtrate is assayed for urea by colorimetry with *p*-dimethylaminobenzaldehyde in acidic solution.

3. The method is applicable when 0.15 mg. to 1.6 mg. of urea is present in 5 ml. of the deproteinized filtrate.

The author is much indebted to Prof. S. Utzino, M. D., for his constant guidance, and Dr. T. Murachi for his invaluable suggestions.

REFERENCE

(*1*) Hunter, A., and Dauphinee, M. A., *Proc. Roy. Soc. London*, **97B**, 209 (1924); *J. Biol. Chem.*, **85**, 627 (1929)

(*2*) Hunter, A., and Pettigrew, J. B., *Enzymologia*, **1**, 341, (1936)

(*3*) Van Slyke, D. D., and Archibald, R. M., *J. Biol. Chem.*, **165**, 293 (1946)

(*4*) Everett, K. V., and Phyllis, R., *J. Biol. Chem.*, **162**, 325 (1945)

(*5*) Watt, G. W., and Chrip, J. D., *Anal. Chem.*, **26**, 452 (1954)

(*6*) Adams, R., and Colemann, G. H., *Org. Syntheses*, coll. vol. **1**, 208 (1932)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

STUDIES ON EGG YOLK PROTEINS

III. PREPARATION AND PROPERTIES OF TWO MAJOR LIPOPROTEINS OF THE EGG YOLK, α- AND β-LIPOVITELLIN

BY HIROSHI SUGANO

(*From the Department of Chemistry, Faculty of Science, Niigata University, Niigata*)

(Received for publication, August 5, 1957)

The works of Alderton, Fevold, and Lausten (*1*, *2*) gave an evidence that the former lipovitellin is not homogeneous. They obtained two preparations of lipoprotein from the former lipovitellin. In spite of the differences in composition and method of preparation, Alderton and Fevold (*1*) have retained the name lipovitellin for one lipoprotein they have isolated. Another lipoprotein has been termed lipovitellenin by Fevold and Lausten (*2*).

In the previous report (*3*), the author has made clear that both of these lipoprotein preparations are not homogeneous electrophoretically and, particularly, the lipovitellin of Alderton and Fevold is an excessive mixture consisted of several components, involving total phosvitin of the yolk and a considerable amount of β-lipovitellin which is a major component of the lipovitellenin.

Lipovitellin has been the name applied to preparations that represented essentially the total lipoprotein of the yolk (*4*). Therefore, in the previous report (*3*), the author has used the names, α- and β-lipovitellin, to represent the two electrophoretic components corresponded to two major lipoproteins of the yolk. By using these terms the electrophoretic compositions of the lipovitellin of Alderton and Fevold and the lipovitellenin of Fevold and Lausten have been given (*3*).

In the present paper, details of the isolation procedures and some chemical and physical properties of α- and β-lipovitellin are described. These lipoproteins represent about 75 per cent of the water-insoluble part of the yolk proteins. The alcohol-extractable lipid of the yolk proteins can be explained almost satisfactorily by the lipid contents of these lipoproteins. Some devices have been performed particularly in order to reduce the isolation process as briefly as possible and to be able to prepare phosvitin, α-, and β-lipovitellin simultaneously and with good yields from the same yolk material.

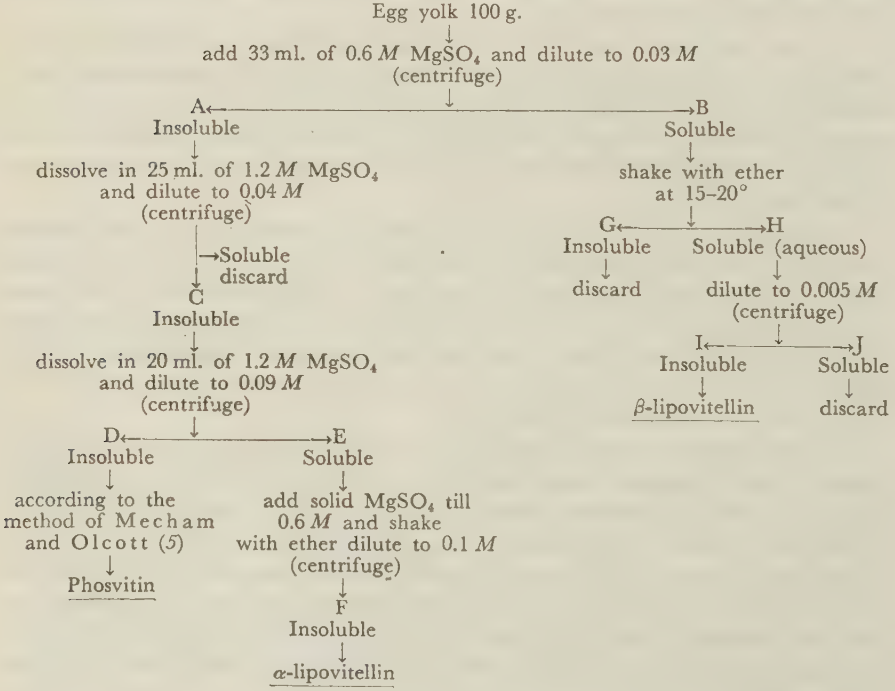
EXPERIMENTAL AND RESULTS

Isolation of α-Lipovitellin

Yolks of fresh eggs were washed in tapwater and rolled on filter paper to free them from adhering white. Chalazae were cut off. The yolk membranes were then punctured and the contents allowed to drain out. To 100 g. of yolk contents were added 33 ml. of 0.6 *M* magnesium sulfate solution and the mixture was stirred vigorously, but without producing foam, for half an hour. Water then was added slowly untill the concentration of magnesium sulfate became 0.03 *M*, the mixture being stirred as before. After two hours at room temperature the produced precipitate was collected by centrifugation at 25,000 r.p.m. for forty minutes (A, Scheme 1) and the soluble fraction (B, Scheme 1) was decanted. The precipitate was dissolved in 25 ml. of 1.2 *M* magnesium sulfate with stirring. After an hour* at room temperature the solution was diluted to 0.04 *M* by the slow addition of water. After three hours the produced precipitate (C, Scheme 1) was collected by centrifugation. The precipitate was again dissolved in 20 ml. of 1.2 *M* magnesium sulfate and after an hour the solution was diluted to

SCHEME 1

Preparation of α- and β-Lipovitellin from Egg Yolk



---

* The solution can be diluted to 0.04 *M* after standing overnight at room temperature.

0.09 *M*. After eighteen hours at room temperature the soft sticky precipitate was collected by centrifugation (D, Scheme 1). This fraction was provided to isolate the phosvitin according to the method of Mecham and Olcott (*5*). To the supernatant (E, Scheme 1) was added solid magnesium sulfate to a concentration of 0.6 *M**. This solution then was extracted thoroughly with ether at room temperature to remove free lipid. After standing overnight, the aqueous layer was drawn off and centrifuged to clear. The solution was then diluted to 0.1 *M* and after two hours the produced precipitate was collected by centrifagation. The precipitate was washed twice centrifugally with water (F, Scheme 1)**. The yield of α-lipovitellin was about 1.1 g. in dry weight.

Isolation of β-Lipovitellin

The soluble fraction (B, Scheme 1) was shaken with ether at 15–20° untill the ether layer became colorless. After standing overnight, three layers separated: an ether layer, an aqueous layer, and an intermediate layer of precipitate (G, Scheme 1) between the other two. The aqueous layer (H, Scheme 1) was drawn off and diluted to 0.005 *M* by the addition of water. After standing overnight at room temperature, the supernatant was decanted and the precipitate was collected by centrifugation and washed once centrifugally with water (I, Scheme 1). Trace amount of the component other than β-lipovitellin was sometimes still found in the I fraction. In such a case, the trace could be removed easily by one time reprecipitation. Namely, the I fraction was dispersed in a large quantity of water, dissolved by careful addition of 0.1 *N* sodium hydroxide, and precipitated again by careful addition of 0.1 *N* sulfuric acid. The yield of β-lipovitellin was about 3.6 g. in dry weight.

Additional Remarks to the Isolations of α- and β-Lipovitellin

The precipitate A could be collected by the centrifugation at 3,500 r.p.m. for sixty minutes, but, in this case, little more non-β-lipovitellin proteins (including phosvitin) were remained in the soluble fraction B. However, these proteins could be removed as the precipitate G by ether extraction of the soluble fraction B. Accordingly, even if the precipitate A was collected by centrifuging at 3,500 r.p.m., an almost similar result would be obtained.

The α-lipovitellin obtained as the precipitate F showed a slight tailing edge in the electrophoretic pattern, as shown in Fig. 1-c. In order to study whether this unsymmetry is attributed to the contamination by β-lipovitellin, an attempt to obtain products having still lower lipid or phosphorus content was carried out. Namely, a further purified α-lipovitellin was prepared by the procedures as shown in Scheme 2. However, the lipid and phosphorus contents of obtained α-lipovitellin were equal to those of the preparation obtained by the method of Scheme 1 within the range of experimental error. Also, no difference could be found between the electrophoretic patterns of both preparations. It was also found from the lipid content and the electrophoretic pattern that even if the procedure obtaining the insoluble C from the insoluble A was omitted, more than 95 per cent pure α-lipovitellin could be obtained.

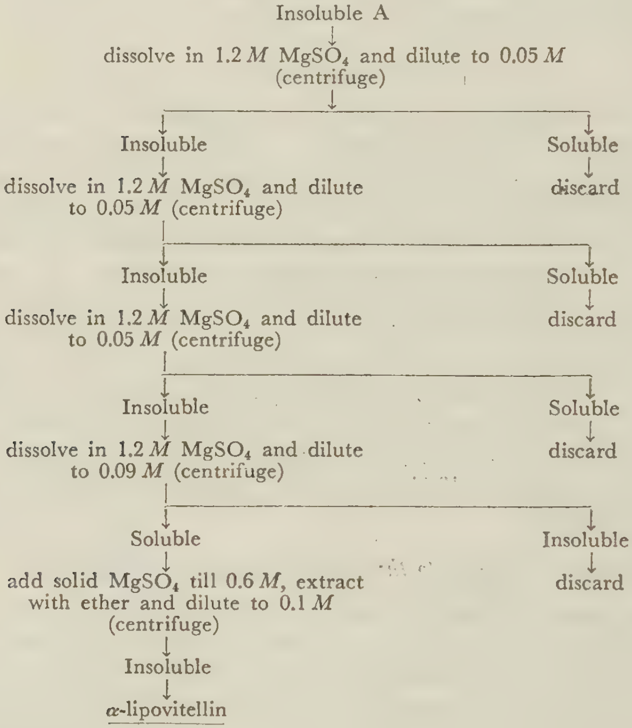
---

* The ether extraction at a concentration below 0.6 *M* produces the precipitate of α-lipovitellin and thus the yield of α-lipovitellin decreases.

** Unless the salt is removed thoroughly by washing, the precipitate can not be dissolved in water by adding alkali.

Scheme 2

Further Purification of α-Lipovitellin


Insoluble A
dissolve in 1.2 M $MgSO_4$ and dilute to 0.05 M (centrifuge)
Insoluble
Soluble
dissolve in 1.2 M $MgSO_4$ and dilute to 0.05 M (centrifuge)
discard
Insoluble
Soluble
dissolve in 1.2 M $MgSO_4$ and dilute to 0.05 M (centrifuge)
discard
Insoluble
Soluble
dissolve in 1.2 M $MgSO_4$ and dilute to 0.09 M (centrifuge)
discard
Soluble
Insoluble
add solid $MgSO_4$ till 0.6 M, extract with ether and dilute to 0.1 M (centrifuge)
discard
Insoluble
α-lipovitellin

When the soluble fraction B was extracted with ether, it is desirable that the temperature is kept over 15°, otherwise a considerable amount of β-lipovitellin would be involved in the insoluble G. In such a case, it had better warm the mixture to about 25° prior to draw off the aqueous layer. If the soluble fraction H were diluted to the concentration below 0.005 M, a very small component (γ-component in Fig. 1-d, having a mobility of −3.5 units) would precipitate together with β-lipovitellin. This component had a property that was precipitated from the soluble fraction H by lowering the temperature below about 10°.

In the first dilution step, dilution to 0.025 M (instead of 0.03 M) was desirable, when the room temperature was over 20°.

Electrophoretic Studies of Isolation Process of α- and β-Lipovitellin*

An electrophoretic pattern of the total yolk protein after ether extraction is shown in Fig. 1-a. And, an electrophoretic pattern of the insoluble A (Scheme 1) is shown in Fig. 1-b. It is evident that this precipitate involves almost all components of the yolk proteins. The electrophoretic composition of this preparation was as follows: phosvitin 19.6, α-lipovitellin 38.5, β-lipovitellin 19.3, and the others 22.6 per cent. Therefore, it could be said that the insoluble A is a similar preparation with the lipovitellin of Alderton and Fevold, which was prepared by centrifuging the egg yolk diluted with two volumes of water, having a following electrophoretic composition: phosvitin 17.0, α-lipovitellin 38.0, β-lipovitellin 27.5, and the others 17.5, per cent (*3*). From the percentage of phosvitin in the insoluble A, it was found that about half quantity of the water-insoluble part of the yolk proteins precipitated as the insoluble A**.

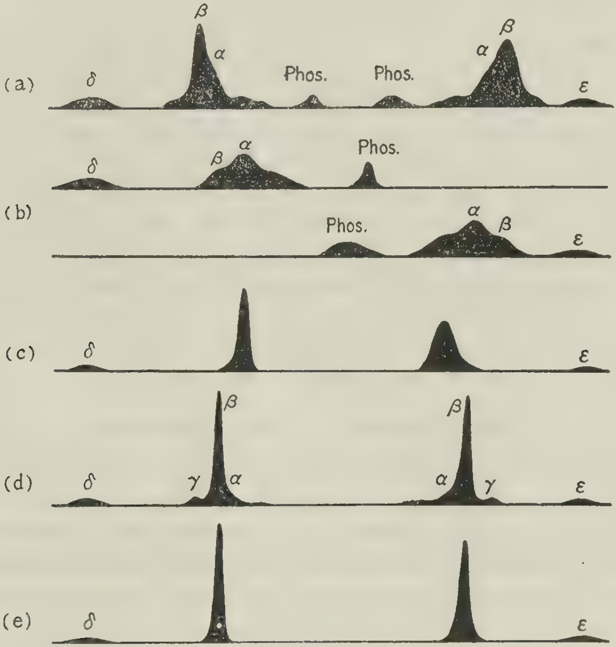


FIG. 1. Electrophoretic patterns of total yolk protein and fractions (carbonate-bicarbonate buffer, pH 9.8 and μ 0.15).

(a) Total yolk protein. (b) The insoluble A fraction. (c) The insoluble F fraction (α-lipovitellin). (d) The soluble B fraction (Livetin fraction was removed by dialyzing against water). (e) The insoluble I fraction (β-lipovitellin).

---

* Electrophoretic experiments were carried out as before (*3*).

** The percentage of phosvitin in the water-insoluble part of the yolk proteins is 9.9 per cent.

Fig. 1-d is an electrophoretic pattern of the fraction that was prepared as follows; namely, to the soluble B was added solid sodium chloride till 10 per cent, extracted with ether untill the ether layer became colorless, and dialyzed against water to separate from the livetin fraction. The electrophoretic composition of this preparation was as follows: α-lipovitellin 8.6, β-lipovitellin 82.2, and the others 9.2 per cent. Therefore, it will be said like the above that this preparation is similar with the lipovitellenin of Fevold and Lausten. On the basis of these results, it could be estimated that about 82 per cent of α-lipovitellin and 19 per cent of β-lipovitellin in the yolk precipitated as the insoluble A fraction.

An electrophoretic pattern of α-lipovitellin obtained as the insoluble F fraction is shown in Fig. 1-c. It is apparent that the components other than α-lipovitellin found in the insoluble A were perfectly removed by the subsequent procedures. It has represented a single peak, while having a slight tailing edge, and had a mobility of -5.3 units at pH 9.8 and μ 0.15.

An electrophoretic pattern of β-lipovitellin is shown in Fig. 1-e. The components having larger mobilities than β-lipovitellin was removed as the insoluble G and γ-component entered in the soluble J fraction. This γ-component is presumed to be a lipoprotein because of its solubility property similar to β-lipovitellin*. The γ-component represented 8.0 per cent in Fig. 1-d. Therefore, it corresponded to 3.4 per cent of the water-insoluble part of the yolk proteins. The β-lipovitellin has represented a single peak and a mobility of -4.4 units at pH 9.8 and μ 0.15.

Some Properties of α- and β-Lipovitellin

*Phosphorus and Lipid Contents***—The phosphorus and lipid contents of α- and β-lipovitellin are given in Table I. The α- and β-vitellin are preparations obtained by alcohol extraction of the respective lipovitellins. About 80 per cent of the phosphorus of β-lipovitellin and only 14 per cent of the phosphorus of α-lipovitellin were attributable to lipid phosphorus.

TABLE I

*Chemical Properties of α- and β-Lipovitellin and Vitellin**

Preparations	Phosphorus	Lipid	Molisch reaction
α-Lipovitellin	0.7 (%)	6–7 (%)	+
α-Vitellin	0.6	0	+
β-Lipovitellin	1.6	37–39	+
β-Vitellin	0.3	0	+

* All values on dry basis.

---

* If so, it will be suitable to be named γ-lipovitellin.

** Phosphorus and lipid contents were determined as before (3).

Carbohydrate—The existences of carbohydrate in these preparations were indicated by Molisch reaction (*6*) (Table I).

Light Absorption—The ultraviolet absorption spectra of α- and β-lipovitellin were similar with that of simple protein. In the visible region, the solutions of α- and β-lipovitellin gave the absorption spectra as shown in Fig. 2. In β-lipovitellin, three maxima were found at about 430, 455, and 490 mμ. In α-lipovitellin, no significant absorption could be found. An ether extract of the soluble B fraction (Scheme 1) gave also three maxima, but their positions somewhat shifted to the short wave length (about 425, 450, and 475 mμ). An ether extract of the soluble E fraction gave also three maxima at about 425, 450, and 475 mμ. It is well known that the color of the yolk is due to carotinoids (xanthophyll and β-carotene) (*7*). By comparing with the absorption spectra of carotinoids (*8*), it is inferable that three maxima are due to the presence of carotinoids, although they are known to be quite strongly affected by the solvent (*8*, *9*). Oncley, Gurd, and Melin (*10*) reported from absorption measurements that human serum β-lipoprotein containd about 0.02 per cent (0.5 moles/mole β-lipoprotein) carotinoids as β-carotene. However, the amount of carotinoids contained in β-lipovitellin is far less than in serum β-lipoprotein, because the absorption due to carotinoids is considerably lower than in β-lipoprotein in spite of higher protein concentration (*10*). In Fig. 2, *a* and *b* are the absorption curves of β-lipovitellin prepared under the different conditions of ether extraction: namely, in the case of *a* the β-lipovitellin was obtained by extracting the soluble B fraction untill the ether layer became colorless, and in the case of *b* the β-lipovitellin obtained like the above was dispersed in a small quantity of water and further extracted with ether. It was thus understood that the slight amount of carotinoids contained in β-lipovitellin decreased to only a

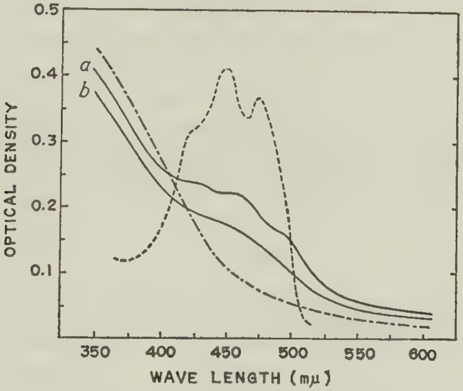


FIG. 2. Absorption spectra of α- and β-lipovitellin and ether extract. —·—·—, α-lipovitellin, 2.5 per cent solution, pH 9.5; ———, β-lipovitellin, 2.5 per cent (*a*), 2.4 per cent (*b*) solution, pH 8.5 (see text); - - - - -, ether extract of the soluble B fraction.

trace in the case of *b**.

Solubility—Both lipovitellin preparations were insoluble to water of neutral pH region, but could be dissolved easily by adding alkali. When the wet precipitates were suspended in water, and to the suspensions 0.1 *N* sodium hydroxide was carefully added, the solutions were obtained at pH 9.3 to 9.6 with α-lipovitellin and at pH 8.0 to 8.5 with β-lipovitellin. These solutions were transparent and not viscous. When the pH of these solutions were lowered by adding 0.1 *N* sulfuric acid, the precipitates were formed at pH 7.5 to 7.8 with α-lipovitellin and at pH 6.5 to 7.0 with β-lipovitellin. Lowering the pH further, the precipitates dissolved again at pH 4.5 to 5.0 with the both. The β-lipovitellin dissolved also easily in 10 per cent sodium chloride saturated with ether and formed a transparent solution. From the preliminary experiments concerning the relationships of pH and ionic strength to the solubility of β-lipovitellin, it was found that at the pH region from about 5.0 to 9.0 the solubility of β-lipovitellin became larger with the increase of ionic strength, but at the pH above 9.0 and below 5.0 the solution increased the turbidity with the increase of ionic strength.

DISCUSSION

A lipoprotein that is similar in composition to β-lipovitellin has been obtained by Shepard and Hottle (*11*). These workers precipitated the major portion of the yolk lipoprotein by dilution of the yolk with two volumes of 0.85 per cent sodium chloride in the presence of ether. The supernatant solution was extracted further with ether, and a lipoprotein was precipitated when the dissolved ether was removed from the aqueous solution. The lipoprotein contained 39 per cent bound lipid and 1.16 per cent phosphorus. It has been confirmed electrophoretically in this laboratory that this lipoprotein is the same component to β-lipovitellin. However, the method used by these workers is not suitable to obtain β-lipovitellin with good yield.

Recently, Vandegaer, Reichman and Cook (*12*) have carried out an ultracentrifugal study of the yolk. The ultracentrifugal examinations of diluted egg yolk indicated three components separable by their sedimentation properties: one rising (R) and two sedimenting (S_1 and S_2). Two of these (R and S_1) were lipoproteins and their alcohol-extractable lipid represented about 40 and 10 per cent, respectively. It is assumed that these two lipoproteins correspond to β- and α-lipovitellin obtained here, respectively.

SUMMARY

1. The preparation method of α- and β-lipovitellin, which are two major lipoproteins of the egg yolk, was described.

---

* In this case, the lipid content of β-lipovitellin decreased also to about 35 per cent.

2. Both of these preparations represented single peak in electrophoretic analysis and had the mobilities of −5.3 and −4.4 units at pH 9.8 and μ 0.15, respectively.

3. Some chemical and physical properties (lipid and phosphorus contents, carbohydrate, light absorption, and solubility) were described.

The author wishes to express his thanks to Miss. I. Watanabe for her technical assistance in carrying out the experiments.

REFERENCES

(*1*) Alderton, G., and Fevold, H. L., *Arch. Biochem.*, **8**, 415 (1945)
(*2*) Fevold, H. L., and Lausten, A., *Arch. Biochem.*, **11**, 1 (1946)
(*3*) Sugano, H., *J. Biochem.*, **44**, 205 (1957)
(*4*) Warner, R. C., in "The Proteins" ed. by Neurath, H., and Bailey, K., Academic Press Inc., New York, Vol. **1**, Part **A**, 1954
(*5*) Mecham, D. K., and Olcott, H. S., *J. Am. Chem. Soc.*, **71**, 3670 (1949)
(*6*) Molisch, H., Mikrochemie der Pflanze, Gustav Fischer Jena, 2 Aufl., S. 128, 1921
(*7*) Romanoff, A. L., and Romanoff, A. J., The Avian Egg, John Wiley & Sons, Inc., New York, Chap. 7, 1949
(*8*) Devine, J., Hunter, R. F., and Williams, N. E., *Biochem. J.*, **39**, 5 (1945)
(*9*) Gillam, A. E., and Stern, E. S., An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry, Edward Arnold (Publishers) Ltd., London, Chap. 14, 1955
(*10*) Oncley, J. L., Gurd, F. R. N., and Melin, M., *J. Am. Chem. Soc.*, **72**, 458 (1950)
(*11*) Shepard, C. S., and Hottle, G. A., *J. Biol, Chem.*, **176**, 349 (1949)
(*12*) Vandegaer, J. E., Reichmann, M. E., and Cook, W. H., *Arch. Biochem.*, **62**, 328 (1956)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

THE EFFECTS OF BROMINATION ON THE ULTRAVIOLET ABSORPTION SPECTRA OF THE PURINE AND PYRIMIDINE BASES AND THE NUCLEOSIDES

BY TAKESHI SUZUKI AND EIJI ITO

(From the Department of Chemistry, Faculty of Science, University of Hokkaido, Sapporo)

(Received for publication, September 20, 1957)

In recent years, the presence of a variety of free ribonucleotides have been showed in biological materials. Among the methods to identify their base components, the measurement of their particular ultraviolet absorption spectra is most frequently used. Several authors have made use of the change in the ultraviolet absorption caused by the bromine treatment for the identification of uracil derivatives. Caputto *et al.* (*1*) carried out bromination directely on uridine diphosphate glucose and Bergkvist *et al.* (*2*) on uridylic acid. On the other hand, Hamer *et al.* (*3*) described the changes owing to bromination of pyrimidine bases and those owing to reduction of pyrimidine and purine bases.

The present report describes the behavior of the ultraviolet absorption spectra of adenine, guanine, uracil and cytosine and their ribonucleosides after the bromine treatment under various conditions. Finally it is concluded that the treatment may be available for the identification of isolated bases and nucleosides and for the approximate estimation of base components in mixtures.

METHODS

Commercial adenine (Zellstoff-fabrik Waldhof) and synthetic uracil were used as the sample. The samples of other bases and nucleosides were prepared from yeast. Some sample of guanosine was kindly supplied by Prof. Mizuno, Hokkaido University. These samples were tested for impurities by the spectra measurements and the paper chromatography.

Unless otherwise noted, bromination was carried out as follows: To a test tube containing 3 ml. of a sample solution in 0.1 *N* hydrochloric acid, one drop of diluted or undiluted bromine water was added. The stoppered test tube was immersed in a constant temperature bath at 50° or allowed to stand at room temperature for the desired time. After aerating for 10 minutes, the ultraviolet absorption was measured by a model DU Beckman spectrophotometer in a cuvette with a light path of 1 cm.

RESULTS

Influence of pH on Bromination—By bromination in a weak alkaline solution, all the tested samples lost their characteristic absorption maxima in the ultraviolet region. As pH of the test solution decreased, the rate of the diminution of the optical density at their absorption maxima was decreased. As the most suitable condition, bromination was performed in 0.1 *N* hydrochloric acid solution and the absorption measurement was carried out without further pH adjustment. All the samples were limited their concentration to below 1 in regard to the optical density at their respective

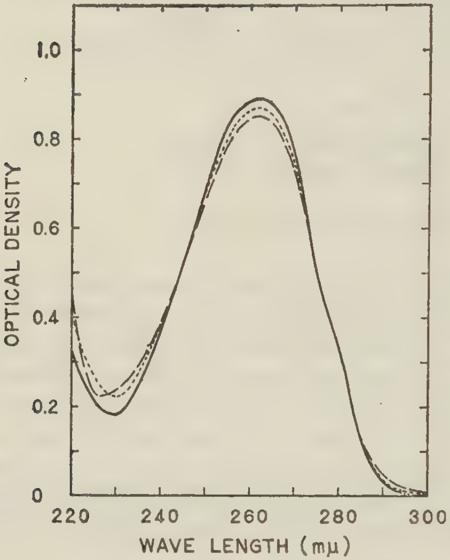


FIG. 1. Changes in the absorption curves of adenine.

To 3 ml. of adenine solution (6.7×10^{-5} *M*) in 0.1 *N* hydrochloric acid was added one drop of bromine water of various concentration. After 10 minutes at room temperature, the solutions were aerated for 10 minutes and the ultraviolet absorption spectra were measured. ——: no bromine water added, — —: undiluted bromine water added, ······: 5 fold diluted bromine water added.

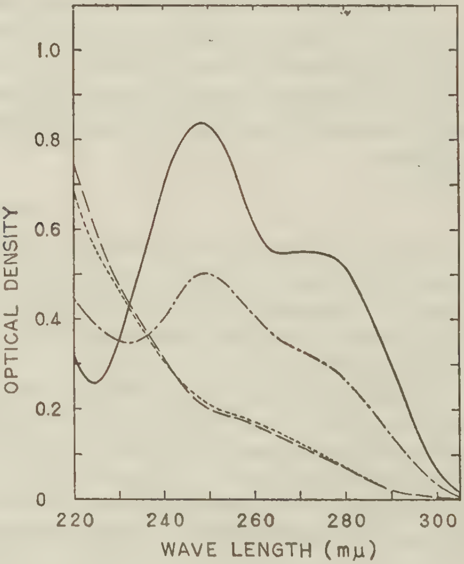


FIG. 2. Changes in the absorption curves of guanine.

Concentration of guanine solution was 1.1×10^{-4} *M*. The experimental procedure was similar to in Fig. 1. ——: no added, — —: undiluted, ······: 5 fold diluted. —·—: 20 fold diluted bromine water added.

absorption maxima in order to avoid a confusion due to a intermedial bromination reaction.

Bromine Treatment of Bases—The typical results are given in Figs. 1 to 4.

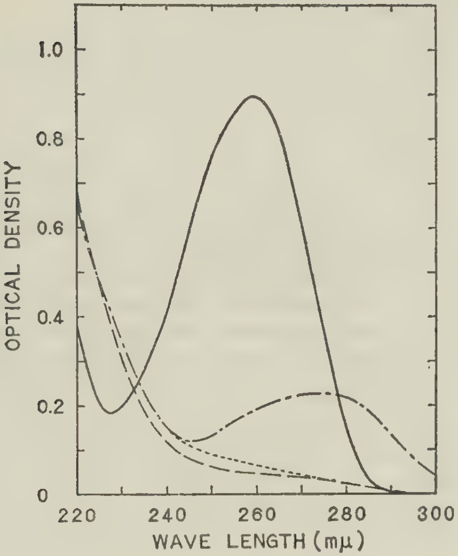
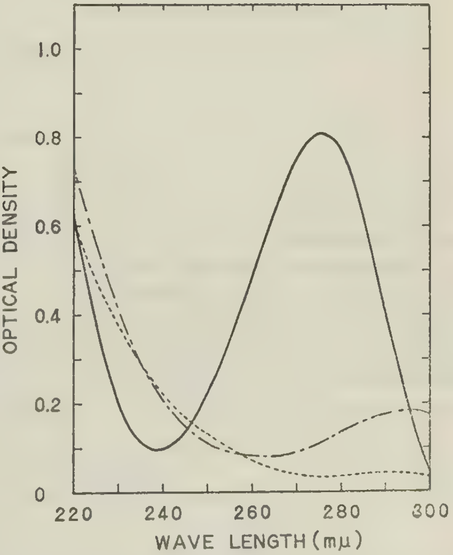


FIG. 3. Changes in the absorption curves of uracil.

Concentration of uracil solution was $1.1 \times 10^{-4} M$. The experimental procedure was similar to in Fig. 1. ——: no added, — —: undiluted, ⋯⋯: 5 fold diluted, —·—: 20 fold diluted bromine water added.

FIG. 4. Changes in the absorption curves of cytosine.

Concentration of cytosine solution was $8.1 \times 10^{-5} M$. The experimental procedure was similar to in Fig. 1. ——: no added, ⋯⋯: 5 fold diluted, —·—: 20 fold diluted bromine water added.



Adenine showed a slight diminution of the absorption after the treatment with one drop of 5 fold diluted bromine water for 10 minutes and extent of the decrease was doubled when bromination was carried out with one drop of undiluted bromine water. On the other hand, the spectra of guanine, uracil and cytosine showed a total sbsence of their particular absorption maxima under the latter condition. When guanine was brominated by the addition of one drop of 20 fold diluted bromine water, a somewhat lowered but characteristic absorption curve was obtained. Whereas, immediately after the addition of diluted bromine water to uracil and cytosine solution,

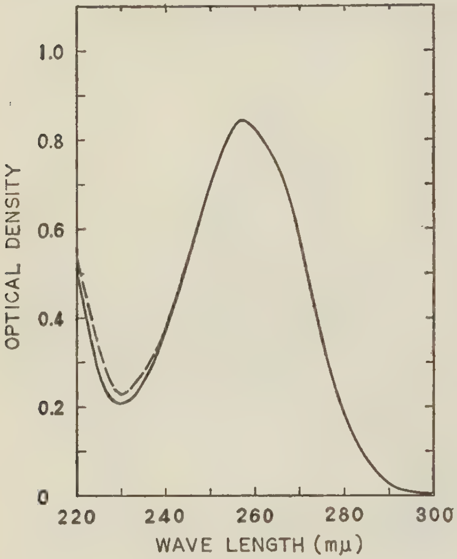


FIG. 5. Changes in the absorption curves of adenosine.

Concentration of adenosine solution was 5.6×10^{-5} *M*. The experimental procedure was similar to in Fig. 1. ——: no added, — —: undiluted bromine water added.

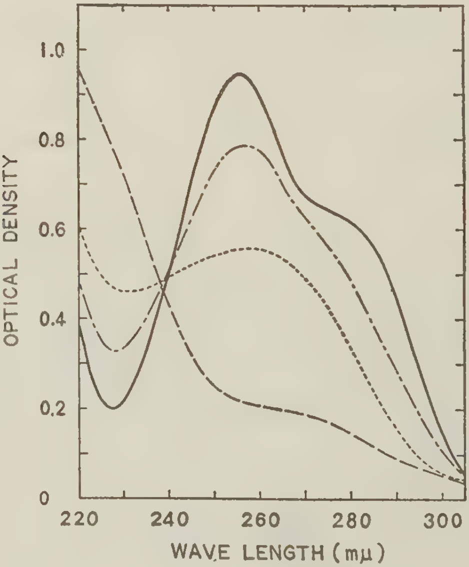


FIG. 6. Changes in the absorption curves of guanosine.

Concentration of guanosine solution was 7.7×10^{-5} *M*. The experimental procedure was similar to in Fig. 1. ——: no added, — —: undiluted, ······: 5 fold diluted, —·—: 20 fold diluted bromine water added.

the peaks of the absorption curves peculiar to them vanished and new low peaks appeared at 280 mμ and 295 mμ, respectively. Here, it shoud be noted that only cytosine showed a extensive elevation of the absorption curve at the shorter wavelength as the absorbancy was estimated in 2 N hydrochloric acid solution after the incubation with bromine water at 50°. The absorption curves of guaninc, uracil and cytosine after the treatment with 5 fold

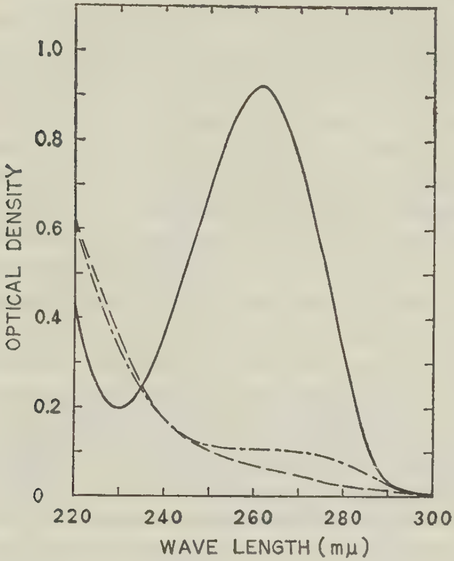
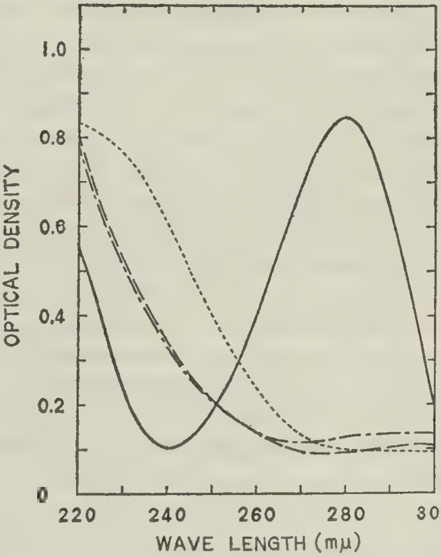


FIG. 7 Changes in the absorption curves of uridine.

Concentration of uridine solution was 9.3×10^{-5} M. The experimental procedure was similar to in Fig. 1. ——: no added, — —: undiluted, —·—: 20 fold diluted bromine water added.

FIG. 8. Changes in the absorption curves of cytidine.

Concentration of cytidine solution was 6.4×10^{-5} M. The experimental procedure was similar to in Fig. 1 except that one sample was treated with one drop of undiluted bromine water at 50° for 10 minutes and the ultraviolet absorption spectrum was measured in 2 N hydrochloric acid solution. ——: no added, — —: undiluted, ⋯⋯: undiluted, 50°, —·—: 20 fold diluted bromine water added.



diluted bromine water crossed those of intact bases at 232.5 mμ, 233.5 mμ and 246.5 mμ, respectively. It was proved that the lowerings of the absorption curves are proportional to the concentration of samples, while that the crossing wavelengths are unconcerned to the concentration.

Bromine Treatment of Nucleosides—As seen in Figs. 5 to 8, nucleosides showed behaviors similar to those of their respective base components. Adenosine was not affected by the incubation with one drop of bromine water at 50° or at room temperature for 10 minutes. In contrast to uridine and cytidine, which lost almost completely their absorption maxima, guanoine gave an about 15 per cent lowered, particular absorption curve, when they were treated with one drop of 20 fold diluted bromine water at room temperature for 10 minutes. The absorption curves of guanosine, uridine and cytidine after bromination with one drop of undiluted bromine water intersected the absorption curves of intact ones at 239 mμ, 235 mμ and 251 mμ, respectively.

Qualitative Test—The feature of changes in the absorption curves of nucleosides and bases caused by bromination will be utilized to identify them and to determine their purity. For the qualitative detection of each nucleosides in a mixture, the following condition was prefered. To 3 ml. of a sample solution, which was adjusted the concentration to give an maximum optical density less than 1 and contained 0.1 N hydrochloric acid, one drop of 20 fold diluted bromine water was added. After 10 minutes, the test solution was aerated for the same time and measured the optical density, giving absorption curve I. Another 3 ml. of the sample solution was treated with one drop of undiluted bromine water, giving curve II. The presence of adenosine was demonstrated as a remaining maximum or a shoulder at about 260 mμ of curve II, and guanosine was detected as lowering of curve I to curve II. The difference between the absorption curve of the intact sample and curve I, if the portion corresponding to guanosine was subtracted, was attributed to the presence of uridine as measured at 251 mμ and to the presence of cytidine at 291 mμ. The presence of cytosine derivatives may be also indicated by brominating at 50° with one drop of undiluted bromine water and measuring the rising of the absorption curve at shorter wavelengths.

Approximate Estimation—For the quantitave analysis of a mixture of nucleosides, bromination was performed with one dorp of undiluted bromine water per 3 ml. at room temperature for 10 minutes in 0.1 N hydrochloric acid. Sets of the wavelengths selected for the estimation of components are somewhat arbitrary. In order to achieve a high sensitivity, a complicated calculation will be required. In natural substances, but, some amounts of ultraviolet absorbing impurities are often present, which may be affected by bromination, and occurrence of an error may be unevitable. Accordingly, in the present paper, convenient wavelengths to the approximate estimation, 247 mμ, 251 mμ, 262 mμ, 276 mμ and 291 mμ were selected. The values of decrease in the absorbancy due to the bromine treatment are showed in Table I as expressed in the molar extinction coefficient. The calculation of values of molar extinction coefficient were based on molar absorbancy of 15.4×10^3 for adenylic acid at 259 mμ, pH 7 (*4*); 12.3×10^3 for guanosine at 256 mμ, pH 1 (*5*); 10.0×10^3 for uridylic acid at 262 mμ, at pH 7 (*6*); 13.0×10^3 for 5′-cytidylic acid at 280 mμ, pH 2 (*7*).

TABLE I

Decrease in the Molar Extinction Due to the Bromine Treatment

Compound	$\Delta E \times 10^{-3}$			
	247 $m\mu$	251 $m\mu$	276 $m\mu$	291 $m\mu$
Adenosine	0	0	0	0
Guanosine	6.4	8.4	6.2	4.4
Uridine	5.1	6.7	5.2	0.1
Cytidine	−1.6	0	11.1	7.6

Fortunately, the value at 247 mμ is nearly equal to one at 276 mμ for either of uridine and guanosine, and so, the following approximate equations are available for the rough estimation of nucleosides.

$$\Delta D_{276} - \Delta D_{247} = 12.7 \times 10^3 \times C_{\text{cytidine}}$$

$$\Delta D_{291} = 4.4 \times 10^3 \times C_{\text{guanosine}} + 7.6 \times 10^3 \times C_{\text{cytidine}}$$

$$\Delta D_{251} = 6.7 \times 10^3 \times C_{\text{uridine}} + 8.4 \times 10^3 \times C_{\text{guanosine}}$$

$$D_{262} = 14.3 \times 10^3 \times C_{\text{adenosine}} + 11.2 \times 10^3 \times C_{\text{guanosine}} + 10.0 \times 10^3 \times C_{\text{uridine}} + 7.1 \times 10^3 \times C_{\text{cytidine}}$$

Where, D is the optical density of an intact sample. ΔD's are decrease in the optical densities at the respective wavelengths due to the bromine treatment and C's are the respective concentrations expressed as moles per liter.

The corresponding approximate equations for a mixture of bases are as follows, in this case, bromination was carried out with one drop of 5 fold diluted bromine water per 3 ml.:

$$\Delta D_{276} - \Delta D_{241} = 11.0 \times 10^3 \times C_{\text{cytosine}}$$

$$\Delta D_{286} = 4.7 \times 10^3 \times C_{\text{guanine}} + 6.6 \times 10^3 \times C_{\text{cytosine}}$$

$$\Delta D_{246} = 4.8 \times 10^3 \times C_{\text{uracil}} + 7.7 \times 10^3 \times C_{\text{guanine}}$$

$$D_{260} = 13.0 \times 10^3 \times C_{\text{adenine}} + 8.0 \times 10^3 \times C_{\text{guanine}} + 8.2 \times 10^3 \times C_{\text{uracil}} + 6.0 \times 10^3 \times C_{\text{cytosine}}$$

Calculation of the values were based on molar absorbancy at 260 mμ, pH 1, which are present in the fourth equation (*8*).

The results of an application of this procedure to a mixture of nucleosides are showed in Table II.

TABLE II

Estimation of Base Components in Nucleoside Mixture

Component of mixture	Content		Per cent recovery
	Added	Recovered	
	$M \times 10^5$	$M \times 10^5$	
Adenosine	2.00	1.91	96
Guanosine	2.70	2.73	101
Uridine	3.48	3.58	103
Cytidine	2.06	2.06	100

DISCUSSION

The nucleotides have absorption characteristics practically undistinguishable from their parent nucleosides (*8*). Therefore, it may be expected that behaviors of nucleotides toward bromine treatment are similar to those of respective nucleosides. Actually the above-mentioned procedure was proved to be applicable to nucleotides. In rather many cases, even after some procedures for the fractionation such as the ion exchange chromatography, nucleotides are present in a mixture, on which occasion, the present method will serve a brief test for the identification or the estimation of components. Furthermore, it may be possible to estimate roughly the base components in a combined form such as in nucleic acid. In an application of the procedure, precaution should be taken against the presence of other bromine-consuming substances. And existence of ultraviolet absorbing impurities, which may be sometimes affected by bromination, causes the most part of an error. In absence of such an impurity, the estimation can be carried out with an error less than 5 per cent. In this paper, changes by bromination in the structure of bases are not touched (*9*).

SUMMARY

Adenine, guanine, uracil and cytosine and their nucleosides were treated with bromine under various conditions, and changes in the ultraviolet absorption spectra were described. On the treatment with diluted bromine water, adenine and adenosine showed a slight or no change in the absorption curve, guanine and guanosine showed a medium change, while uracil, cytosine and their nucleosides lost the absorption maxima completely. These characteristic changes in the absorption curves give a possibility that the procedure is available for the identification of isolated bases and nucleosides and for the approximate estimation of base components in mixtures.

The authors wish to express their sincere gratitude to Prof. Y. Mizuno for his kind supply of some sample and to Prof. N. Takasugi for his kind advices in this work.

REFERENCES

(*1*) Caputto, R., Leloir, L. F., Cardini, C. E., and Paladini, A. C., *J. Biol. Chem.*, **184**, 333 (1950)

(*2*) Bergkvist, R., and Deutsch, A., *Acta Chem. Scand.*, **8**, 1880 (1954)

(*3*) Hamer, D., Waldron, D. M., and Woodhouse, D. L., *Arch. Biochem. and Biophys.*, **47**, 272 (1953)

(*4*) Morell, S. A., and Bock, R. M., Am. Chem. Soc., 126th Meeting, New York, Div. of Biol. Chem. Abstracts, p. 44 c (1954)

(*5*) Pabst Laboratories Circular OR-10, p. 17 (1956)

(*6*) Ploeser, J. M., and Loring, H. L., *J. Biol. Chem.*, **178**, 431 (1949)

(*7*) Volkin, E., and Cohn, W. E., Methods of Biochemical Analysis, ed. by Glick, D., Interscience, New York, Vol. **I**, p. 304 (1954)

(*8*) Beaven, G. H., Holiday, E. R., and Johnson, E. A., The Nucleic Acids, ed. by Chargaff, E., and Davidson, J. N., Academic Press, New York, Vol. **I**, p. 493 (1955)

(*9*) Cohn, W. E., *Biochem. J.*, **64**, 28 p (1956)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

CRYSTALLINE BACTERIAL PROTEINASE

IV. AMINO-TERMINAL AMINO ACID OF CRYSTALLINE BACTERIAL PROTEINASE

By HIROSHI MATSUBARA AND SHIGEHIKO NISHIMURA

(From the Department of Biology, Faculty of Science, University of Osaka, Osaka)

(Received for publication, October 2, 1957)

It has been previously reported (*1*), as a result of investigations on general properties of crystalline bacterial proteinase (BPN′) obtained from culture medium of *Bac. subtilis N′* (*2–6*), that the proteinase has so broad a specificity that it seems to have a different structure in active area from the other animal and neutral proteolytic enzymes, such as trypsin and chymotrypsin. However, its chemical structure is not yet known. In view of investigations on the relation between structure and activity of the enzyme, the first is to determine the terminal groups of the protein. For such an investigation it is naturally necessary to use tne purest sample, but in the case of proteolytic enzyme an attention must be paid to prevent the contamination of amino acids or peptides produced by autodigestion of the enzyme during various treatments as well as obtaining a pure sample. On this point, Desnuelle *et al.* (*7*, *8*) investigated the terminal groups of chymotrypsin and trypsin, and Thompson theose of papain (*9*).

In the present communication it will be shown that the amino (N)-terminal amino acid of BPN′ is alanine alone.

MATERIALS AND METHODS

Bacterial Proteinase—Twice recrystallized bacterial proteinase (BPN′)* prepared according to the method of Hagihara *et al.* (*2*, *3*) was recrystallized twice more and washed with 50 per cent acetone, followed by 45 per cent acetone.

BPN' Treated witth Trichloroacetic Acid Solution—The sample prepared as mentioned above was dissolved in distilled water in an ice bath and precipitated by an equal volume of 1.2 *M* trichloroacetic acid (TCA) solution. The precipitate was washed successively with 0.6 *M* TCA solution, distilled water, acetone, and ether. Then the precipitate was dried *in vacuo* and ground to a fine powder (yield 92 per cent).

Diisopropyl Phosphoryl-Proteinase—As will be shown in the following paper (*10*), crystalline diisopropyl phosphoryl (DIP)** derivative of BPN′ was obtained and the sample recrystallixed three times was used in this work.

---

* This sample was kindly supplied by Nagase Co., Ltd.

** We are indepted to Mr. T. Ikenaka of Akabori Laboratory for the gift of a sample of diisopropyl fluorophosphate (DFP) prepared by him.

Dinitrophenyl Derivatives—Dinitrophenyl (DNP) derivatives of the above three samples were prepared approximately in accordance with the usual method of Sanger (*11*). About 300 mg. of protein dissolved in 5 ml. of 0.8 *M* $NaHCO_3$ solution (pH 8.5) was shaken for 2 hours at room temperature (20 to 30°) together with 0.3 ml. of fluorodinitrobenzene (FDNB) dissolved in 10 ml. of ethanol and the resulting yellow precipitate was washed successively with 66 per cent ethanol, acidic water, water, ethanol or acetone, and ether. The amount of original protein in the DNP-protein thus obtained was estimated by determining the amide content and by the ratio between the weights of the original protein and the DNP-protein formed, *i.e.* 85 to 87 per cent.

Hydrolysis and Extraction—Thirty to fifty mg. of DNP-derivatives were hydrolyzed with 2 ml. of 5.7 *N* HCl (twice distilled) in a sealed tube by heating to 105° for 4, 8, and 16 hours, sometimes for 24 hours. The hydrolysate was diluted with 10 ml. of water and extracted with two 20-ml. and one 10-ml. portions of ether. The combined extract was washed with 5 ml. of water and ether was evaporated. The aqueous layer was concentrated and dried *in vacuo*. When DNP-BPN′ and DNP-Pro* were separately hydrolyzed with 12 *N* HCl for 2 to 4 hours at 105° in order to ascertain the presence of Pro in terminal (*12*), DNP-Pro was recovered in a small amount, but no DNP-Pro was identified from DNP-BPN′. Thus, Pro will be not present as N-terminal group of BPN′.

Identification and Quantitative Determination of DNP-Amino Acids—DNP-amino acids in the ether phase were identified by paper chromatography according to the method of Levy (*13*) after removal of excess dinitrophenol by sublimation (*14*), confirmed by regeneration (*15*), and estimated quantitatively by silica gel method of Sanger (*11*). DNP-amino acids in the aqueous layer were identified by paper chromatography (*16*) and quantitatively estimated by ion exchanger (Amberlite IR-112, H-from) chromatography (*17*).

RESULTS

DNP-Amino Acids Obtained from DNP-BPN′—As the yield of DNP-derivative of active BPN′ was very small, examination was made as to the cause of this undesirability and it was found that the autodigestion of BPN′ occurs on the occasion of dinitrophenylation of BPN′ in ethanol, *i.e.* when BPN′ was shaken under the same conditions as above, except for removing FDNB, the amount of soluble materials in concentrated TCA solution was larger than that from treating under the same conditions and removing only ethanol, which suggests that BPN′ digests itself during dinitrophenylation. Therefore, DNP-amino acids obtained in this case were DNP-derivatives of the terminal groups of peptides or amino acids produced by autodigestion during the treatment, besides those of the original terminal amino acids of BPN′. These DNP-derivatives were compatible with the results of studies on the specificity of BPN′ reported previously (*1*).

One of the results obtained is shown in Table I. DNP-Ala appeared in larger amount compared with other DNP-amino acids as N-terminal amino acid, and thus there is a possibility of Ala being the N-terminal amino acid of BPN′, besides showing the specificity. However, it is uncertain from only this result that Ala is the N-terminal residue of BPN′, and in order to eliminate the autodigestion it is necessary to dinitrophenylate the sample after denaturation with heat, urea, or TCA solution, or after inactivation with suitable inhibitory agents. As one of the denaturing agents TCA solution and one of the inactivating agents diisopropyl fluorophosphate were used in this work.

---

* The abbreviations for the amino acid residues suggested by Brand and Edsall (*18*) are used.

TABLE I

DNP-Amino Acids Obtained from DNP-BPN′

DNP-amino acid identified	Moles DNP-amino acid* per mole protein**			
	Time of hydrolysis with acid			
	4 *hr.*	8 *hr.*	16 *hr.*	24 *hr.*
Ala	1.2	1.0	0.79	0.66
Ser	0.97	0.69	0.42	0.38
Asp+Glu	0.58	0.43	0.26	0.23
Gly	0.71	0.36	0.09	—
Val	0.18	0.25	0.44	0.18
Thr	0.13	0.15	0.09	0.09
Arg (α-)***	+	+	+	+

* These values are uncorrected for hydrolytic breakdown.

** The molecular weight of BPN′ was assumed to be 30,500 ±1,000 based on the results of light scattering and monomolecular film measurements.

*** Quantitative data are not shown and only the presence of Arg (α-) is indicated by the sign +.

DNP-Amino Acids Obtained from DNP-Derivatives of BPN′ Treated with TCA Solution—When the DNP-derivatives of BPN′ treated with TCA solution were hydrolyzed with acid, DNP-Ala was mainly found in ether phase together with small amounts of DNP-Ser, -Thr, -Asp, and -Glu. DNP-Ala was recognized as the DNP-derivative of Ala by regeneration treatment and paper chromatography (butanol 4 : glacial acetic acid 1 : water 5). In the aqueous layer ε-DNP-Lys and α-DNP-Arg were found, but the amount of α-DNP-Arg was variable and non-stoichiometric, and an unknown substance, which colors red on only making alkaline in Sakaguchi reaction (*19*), was contaminated in the region of α-DNP-Arg; α-DNP-Arg might be a substance contaminated in the preparation of BPN′. The quantitative estimation of their amount is shown in Table II. When the molecular weight of BPN′ was assumed th be 30,500*, Ala was found to be present as N-terminal amino acid in one mole per mole of BPN′. ε-DNP-Lys was found to be present in about 10 moles per mole of BPN′.

---

* This value was concluded from the results of light scattering experiments on BPN′ and monomolecular film on DIP-BPN′, which were measured by Dr. M. Iwatsubo and Mr. M. Ikeda, respectively.

TCA treatment might give some effect on the N-terminal residue of the protein and therefore, another treatment, DFP-treatment, was adopted.

TABLE II

DNP-Amino Acids Obtained from DNP-Derivatives of BPN′ Treated with TCA Solution

No. of experiment	DNP-amino acid identified	Moles DNP-amino acid* per mole protein**		
		Time of hydrolysis with acid		
		4 hr.	*8 hr.*	*16 hr.*
1	Ala	0.68	1.0	0.93
	Glu+Thr	0.02	0.04	0.03
	Asp+Ser	0.05	0.05	0.03
2	Ala	0.94	0.90	0.98
	Glu+Thr	0.06	0.06	Trace
	Asp+Ser	0.06	0.07	Trace
	Arg (α-)	0.49		
	Lys (ε-)	10.8		
3	Ala	1.1	0.97	
	Arg (α-)		0.54	0.33
	Lys (ε-)		9.0	10.3

* These values are corrected for hydrolytic breakdowns from Table III for Ala and from literature (*11*) for other DNP-amino acids.

** The molecular weight of BPN′ was the same in Table I.

TABLE III

Recovery Factor of DNP-Ala during Acid Hydrolysis

BNP-Ala	DNP-BPN′ treated with TCA solution	BPN′	Time of hydrolysis	DNP-Ala recovered	Recovery factor
(*μg.*)	(*mg.*)	(*mg.*)	(*hr.*)	(*μg.*)	(*%*)
181	—	—	4	146	81
181	—	—	8	130	72
181	—	—	16	119	66
181	50.8	—	8	128	70
181	—	39.2	8	87	48

DNP-Amino Acids Obtained from DNP-Derivative of BPN′ Treated with DFP—Only DNP-Ala was obtained from DNP-derivative of DIP-BPN′ with acid hydrolysis. Therefore, this result agreed with that of TCA-treated sample. α-DNP-Arg was not found in detectable amount.

Recovery Factor of DNP-Ala during Acid Hydrolysis—About 180 μg. of DNP-Ala was hyrolyzed with 5.7 N HCl at 105° for 4, 8, and 16 hours in the presence and absence of DNP-BPN′, and the resulting hydrolysate was treated as mentioned above. Table III shows the result, which was used for the calculation of the amount of terminal Ala in Table II. Other DNP-amino acids were calculated from the data in literature (*11*).

DISCUSSION

It has been shown from the result of these experments that in determining the structure of proteolytic enzymes, it is necessary to avoid autodigestion during the course of various treatments and it is very useful for this purpose to treat the sample with TCA solution or appropriate inhibitory agents. However, it is considered that the terminal groups, which are not found in active form of the protein, have been newly exposed when the protein was treated with some denaturing agents (*20*). Ala was found as the N-terminal residue of BPN′ with TCA treatment and also found in highest value in the autodigestion experiment, and further as the N-terminal amino acid of DIP-BPN′, which will be shown to be native in the following paper. Therefore, it will be suggested that the N-terminal residue of this proteinase was not affected by TCA treatment and did not react with DFP, and it follows that Ala may be present as the terminal amino acid of native BPN′. On the other hand, there is no possibility for supposing Arg as the N-terminal group of BPN′. Therefore, BPN′ consists of one polypeptide chain having the N-terminal group of Ala. Furthermore, when BPN′ oxidized with performic acid was examined by electrophoresis at pH 7.0, only one peak was found, which may support the conclusions reached above. In addition to these methods, it might be reasonable to adopt the method of Levy (*13*), in which dinitrophenylation was effected without ethanol, but in this work it could not unfortunately be used.

Although Desnuelle *et al.* (*7*, *8*) stated that the peptides bound to chymotrypsin and trypsin after their activation were found in analyses of terminal groups of crytalline materials, it may also be considered to represent peptides produced by autodigestion during analytical process, such as dinitrophenylation. This may also be applied in the analyses of papain (*9*) and α-chymotrypsin (*21*).

The amount of terminal Ala was calculated from the molecular weight based on light scattering and monomolecular film experiments, *i.e.* 30,500± 1,000. Thus from quantitative studies the minimum molecular weight of BPN′ was calculated as 31,300.

SUMMARY

The fluorodinitrobenzene method was applied for the determination of the amino-terminal amino acid of bacterial proteinase (BPN′) in various experiments. Alanine was found to be the single terminal amino acid and

some discussions were made on terminal groups in the case of using TCA solution or DFP as agents preventing autodigestion and also on molecular weight.

Addendum—Recently the work of Ottesen and Schellman (*22*) appeared, in which a crystalline diisopropyl fluorophosphate derivative of subtilisin was prepared and its phosphorus content and free amino end group were decided. The inhibited enzyme contained one atom of phosphorus per mole of protein and consisted of a single polypeptide chain with the N-terminal sequence Ala-Glu.

The authors wish to express their many thanks to Prof. K. Okunuki and Dr. B. Hagihara for their kind advices on this work, to Dr. M. Iwatsubo of Kubo Laboratory and Mr. M. Ikeda of Isemura Laboratory for determination of molecular weight of BPN′ and also to Nagase Co., Ltd for their gifts of source materials of the enzyme.

REFERENCES

(*1*) Okunuki, K., Matsubara, H., Nishimura, S., and Hagihara, B., *J. Biochem.* **43**, 857 (1956)

(*2*) Hagihara, B., *Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954)

(*3*) Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K., *J. Biochem.*, **45**, 185 (1958)

(*4*) Matsubara, H., Hagihara, B., Nakai, M., Komaki, T., and Okunuki, K., *J. Biochem.*, **45**, 251 (1958)

(*5*) Hagihara, B., Nakai, M., Matsubara, H., Komaki, T., Yonetani, T., and Okunuki, K., *J. Biochem.*, **45**, 305 (1958)

(*6*) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., *J. Biochem.* **43**, 453 (1956)

(*7*) Desnuelle, P., Rovery, M., et Fabre, C., *Biochim. Biophys. Acta.* **9**, 109 (1952)

(*8*) Rovery, M., Fabre, C., et Desnuelle, P., *Biochim. Biophys. Acta*, **9**, 702 (1952)

(*9*) Thompson, E. O. P., *J. Biol. Chem.*, **207**, 563 (1954)

(*10*) Matsubara, H. and Nishimura, S., *J. Biochem.*, in press.

(*11*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)

(*12*) Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., Methods of Biochemical Analysis, Interscience publishers, INC., New York, **Vol. II**, p. 371 (1955)

(*13*) Levy, A. L., *Nature*, **174**, 126 (1954)

(*14*) Mills, G. L., *Biochem. J.*, **50**, 707 (1952)

(*15*) Mills, G. L., *Nature*, **165**, 403 (1950)

(*16*) Levy, A. L., and Chung, D., *Anal. Chem.*, **25**, 396 (1953)

(*17*) Seki, T., in Chemistry of the Protein, ed. by Akabori, S., and Mizushima, S., Kyoritsu, & Co., Tokyo, **Vol. IV**, p. 229 (1956)

(*18*) Brand, E., and Edsall, J. T., *Ann. Rev. Biochem.*, **16**, 224 (1947)

(*19*) Sakaguchi, S., *J. Biochem.* 5, 25, 133 (1925)

(*20*) Braunitzer, G., *Biochim. Biophys. Acta.* **19**, 574 (1956)

(*21*) Sakota, N., *J. Biochem.* **42**, 69 (1955)

(*22*) Ottesen, M., and Schellman, C. G., *Compt. rend. trav. lab. Carlsberg, sér. chim.*, **30**, 157 (1957)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

XVIII. SEPARATION AND IDENTIFICATION OF THE DEGRADATION PRODUCTS OF CHOLIC ACID BY STREPTOMYCES RUBESCENS

BY SHOHEI HAYAKAWA*, YASUO SABURI**
AND KEIZO TAMAKI*

(*From the *Department of Biochemistry, Okayama University Medical School, Okayama, and** the First Bacteriological Division, Institute for Infectious Diseases, University of Tokyo, Tokyo*)

(Received for publication, December 10, 1957)

In a previous paper (*1*) it has been demonstrated that all of the seven *Streptomyces* spp. (*2*), utilizing cholic acid as the sole source of carbon, are capable of decomposing cholic acid via an intermediate containing 7α-hydroxy-Δ^4-3-ketone structure. Since the first publication (*3*) of this series, microbiological degradation of the various bile acids by *Streptomyces gelaticus* 1164, one of the above seven cholic acid utilizers, has been investigated in detail and recently possible pathways for the cholic acid degradation and the dehydrocholic acid degradation by this microorganism have been proposed (*4–5*).

S. gelaticus 1164 is able to grow in a synthetic medium containing 7α-hydroxy-3,12-dioxo-Δ^4-bisnorcholenic acid (VI), transformed from cholic acid by the same microorganism, as the sole source of carbon. So it seems that *S. gelaticus* 1164 is able to metabolize this bisnor acid further. *Streptomyces rubescens*, one of the above seven cholic acid utilizers, is however, unable to utilize this bisnor acid. These facts suggest that *S. rubescens* is capable of degrading cholic acid *via* a pathway different from that (*4*) by *S. gelaticus* 1164.

In the present experiment, therefore, in order to study a pathway for the cholic acid degradation by *S. rubescens*, isolation and identification of the degradation products of cholic acid by this microorganism have been performed and the following products have been obtained in forms of free acid or methyl ester: 7α,12α-dihydroxy-3-oxo-Δ^4-cholenic acid (III), 7α-hydroxy-3,12-dioxo-Δ^4-cholenic acid (IV) and 3,12-dioxo-$\Delta^{4,6}$-choladienic acid (V).

From the constitutions of these various intermediates isolated here a possible degradation pathway of cholic acid (I) by *S. rubescens* may be presumed as shown in Fig. 1.

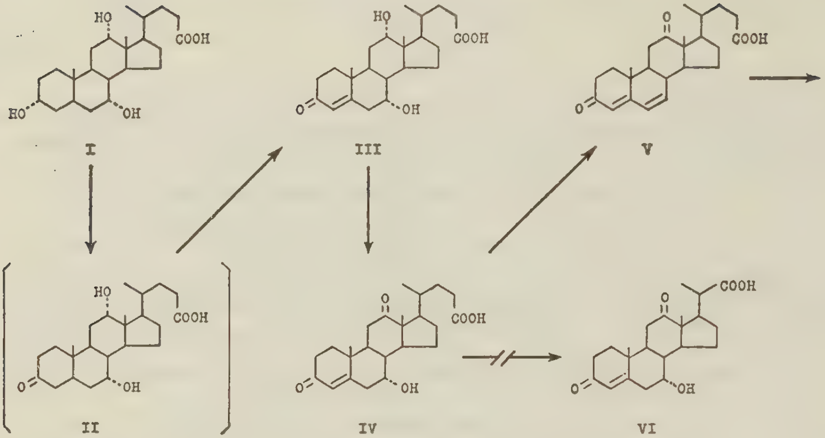


FIG. 1. A proposed pathway for cholic acid degradation by *S. gelaticus*

EXPERIMENTAL

Utilization of 7α-Hydroxy-3,12-dioxo-Δ^4-bisnorcholenic Acid by Seven Cholic Acid Utilizers—20 mg. of 7α-hydroxy-3,12-dioxo-Δ^4-bisnorcholenic acid, prepared from cholic acid by cultivation of *S. gelaticus* 1164 as described in a previous paper (*1*), was dissolved in 0.4 ml. of water containing the calculated amount of sodium bicarbonate to be necessary for neutralizing this acid. The resulting solution was sterilized by filtration through a bacterial filter (Seitz E) and diluted with 20 ml. of an autoclaved Dulaney's mineral solution (*2*) under aseptic conditions. The medium was tubed in 2.5 ml. quantities in the sterilized small test tubes. The seven cholic acid utilizers (*2*) from each Czapeck's slant agar were inoculated in these media and the

TABLE I

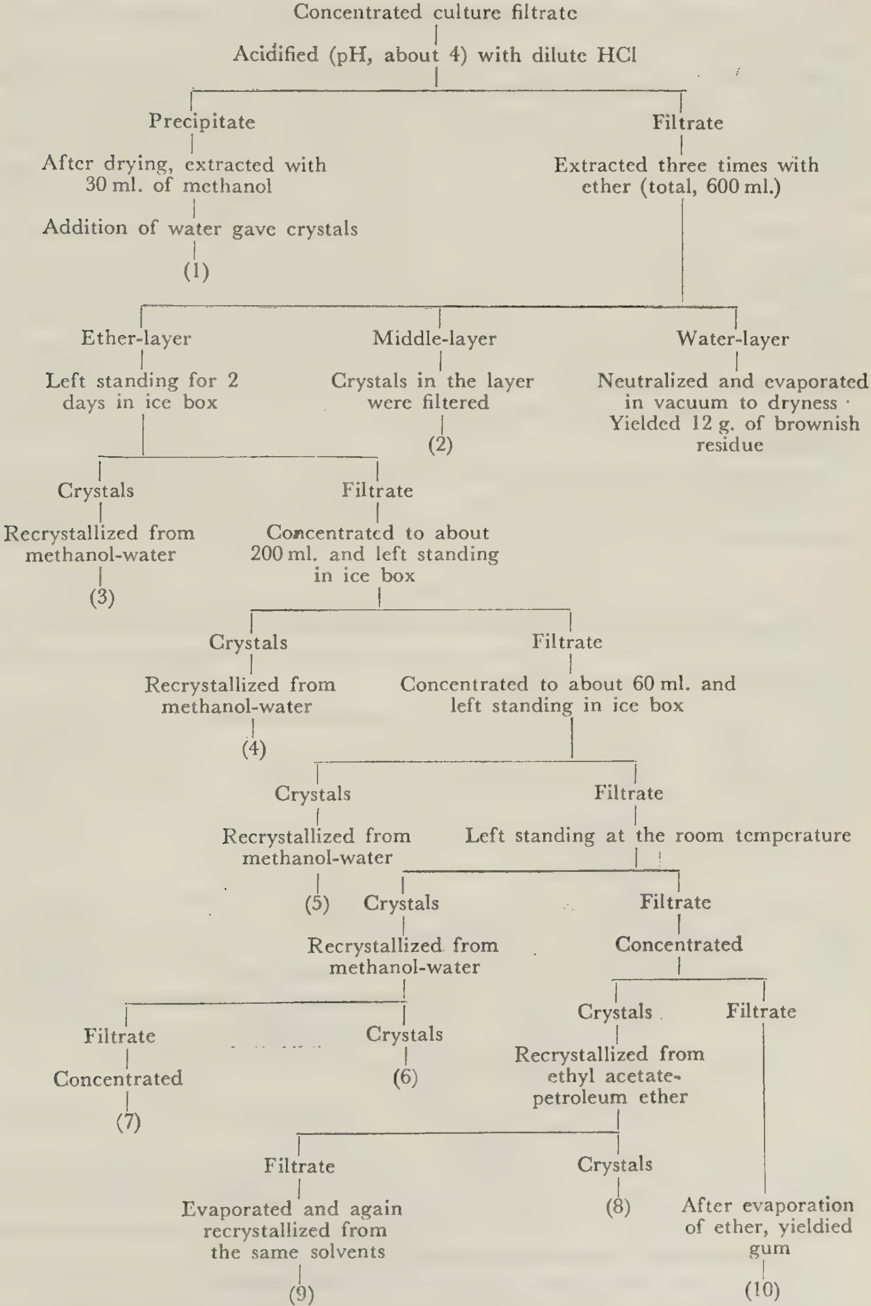
Utilization of 7α-Hydroxy-3,12-dioxo-Δ^4-bisnorcholenic Acid by Seven Cholic Acid Utilizers

Cholic acid utilizers	Period necessary for positive growth (in days)	Degree of the growth after 30 days' incubation*
S. gelaticus 1164	4	++
S. californicus 5119	3	++++
S. flavogriseus 4449	3	+++
S. halstedii 3199	3	++
S. nitrosporeus 0–20	—	—
S. rubescens Z–5–2	—	±
S. scabies 3111	—	±

* Plus signs refer to the degree of the growth: ++～++++ complete growth, ± faint growth, probably no utilization, — non-growth.

SCHEME 1.

Fractionation and Isolation of Cholic Acid Metabolites by S. rubescens.


Concentrated culture filtrate
Acidified (pH, about 4) with dilute HCl
Precipitate
After drying, extracted with 30 ml. of methanol
Addition of water gave crystals
(1)
Filtrate
Extracted three times with ether (total, 600 ml.)
Ether-layer
Left standing for 2 days in ice box
Middle-layer
Crystals in the layer were filtered
(2)
Water-layer
Neutralized and evaporated in vacuum to dryness ·
Yielded 12 g. of brownish residue
Crystals
Recrystallized from methanol-water
(3)
Filtrate
Concentrated to about 200 ml. and left standing in ice box
Crystals
Recrystallized from methanol-water
(4)
Filtrate
Concentrated to about 60 ml. and left standing in ice box
Crystals
Recrystallized from methanol-water
(5)
Filtrate
Left standing at the room temperature
Crystals
Recrystallized from methanol-water
Filtrate
Concentrated
(7)
Crystals
(6)
Filtrate
Concentrated
Crystals
Recrystallized from ethyl acetate-petroleum ether
Filtrate
Evaporated and again recrystallized from the same solvents
(9)
Crystals
(8)
Filtrate
After evaporation of ether, yieldied gum
(10)

Figures given in parentheses refer to the each fraction number in Table II.

inoculated tubes were incubated at 27°. The growth of the seven *Streptomyces* spp. was determined by examining the amount of mycelium grown on the surface of the culture medium for 30 days. The result was given in Table I.

Fractionation and Isolation of Cholic Acid Metabolites by S. rubescens—Sodium cholate was added into the basal mineral solution (*3*) ($(NH_4)_2SO_4$, 2.0 g.; K_2HPO_4, 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeCl_3 \cdot 6H_2O$, 0.01 g.; distilled water, 1,000 ml.) in final concentration of 0.3 g. per 100 ml. and the pH was adjusted to 7.2. 100 ml. of this medium placed in 500 ml. Sakaguchi's flask was autoclaved at 110° for 20 minutes. After inoculation of *S. rubescens*, the inoculated flask was shaken at 27° on a reciprocal shaker (120 r.p.m., 5 cm. amplitude). 9 g. of sodium cholate were used for the present experiment. After incubation for 110 hours, the broth was filtered to remove mycelium. The filtrate (about 2,920 ml.) was neutralized with dilute sodium carbonate solution and the neutralized solution was concentrated to about 300 ml. in vacuum at below 45°. The concentrated culture filtrate was treated as shown in Scheme 1.

Identification of Cholic Acid Metabolites—After the above-obtained fractions were further purified or converted into a derivative, some compounds corresponding to cholic acid metabolites were identified by the mixed

TABLE II

Identification of Cholic Acid Metabolites Obtained from the Fractions in Scheme 1

Properties of each fraction in Scheme 1				Identified substances	m.p. (°C)
No.	Yields (*mg.*)	m.p. (°C)	$\lambda_{max}^{alc.}$ (mμ)*		
1	171	201–205	281 (240)	—**	—
2	408	230–240 (decomp.)	240 (281)	—**	—
3	65	245–250 (decomp.)	240	7α-Hydroxy-3,12-dioxo-Δ^4-cholenic acid	248–252 (decomp.)
4	217	220–232 (decomp.)	240 (281)		
5	100	220–233 (decomp.)	240 (281)	Unidentified	224–226 (decomp.)
6	129	205–207	281	3,12-Dioxo-$\Delta^{4,6}$-choladienic acid	205–208
7	117	154–160	281 (weak)	Unidentified	162–164
8	92	155–158	none***		
9	83	155–158	none***		
10	700	—	—	—**	—

* An inflection is given in parentheses.

** See text for experimental details and identified substances.

*** These showed no characteristic absorption in the region of 220 to 330 mμ.

melting point determination, infrared comparison and microanalysis data. The summary was given in Table II.

Fraction No. 1 was recrystallized from methanol and gave 60 mg. of plates with m.p. 209–210°, but the ultraviolet absorption spectrum of this acid still showed an inflection at 240 mμ with $\lambda_{\max}^{\text{alc.}}$ 281 mμ. After esterification with diazomethane by the usual manner, the ester with m.p. 169–171° was obtained by further purification and identified as methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate.

Analysis Calcd. for $C_{25}H_{34}O_4$: C, 75.34; H, 8.60
Found: C, 75.12; H, 8.72

Fraction No. 2 was esterified with diazomethane, crystallized fractionally from ethyl acetate-petroleum ether and gave two parts which were differed in its ultraviolet absorption maximum. The one with $\lambda_{\max}^{\text{alc.}}$ 281 mμ and m.p. 170–173° was obtained in poor yields, so the identity with an authentic methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate was demonstrated only by mixed melting point determination. The other with $\lambda_{\max}^{\text{alc.}}$ 241 mμ was converted into methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate with m.p. 172–174° by refluxing with methanolic sulfuric acid solution and identified.

Analysis Calcd. for $C_{25}H_{34}O_4$: C, 75.34; H, 8.60
Found: C, 75.26; H, 8.62

As discussed in the previous papers (*5–6*), the above results showed that 3,12-dioxo-$\Delta^{4,6}$-choladienic acid and 7α-hydroxy-3,12-dioxo-Δ^{4}-chlenic acid were probably contained in the fraction No. 2.

Fraction No. 4 was recrystallized from methanol and the melting point raised to 246–250° with decomposition. The purified fraction No. 4 was combined with the fraction No. 3, recrystallized from methanol and gave 7α-hydroxy-3,12-dioxo-Δ^{4}-cholenic acid (*7*) with m.p. 248–252° (decomp.) as shown in Table II.

Analysis Calcd. for $C_{24}H_{34}O_5$: C, 71.61; H, 8.51
Found: C, 71.40; H, 8.29

Fraction No. 5 was recrytallized from methanol, gave 20 mg. of plates with m.p. 224–226° (decomp.) and an absorption peak at 240 mμ, but not identified.

Analysis Calcd. for $C_{24}H_{34}O_5$: C, 71.61; H, 8.51
Found: C, 71.40; H, 8.49

Fraction No. 6 was recrystallized from ethyl acetate-petroleum ether.

Analysis Calcd. for $C_{24}H_{32}O_4$: C, 74.97; H, 8.39
Found: C, 74.62; H, 8.46

Fraction No. 7, No. 8 and No. 9 were combined, recrystallized from ethyl acetate and gave 40 mg. of prisms with m.p. 162–164°, but not identified.

Analysis Calcd. for $C_{22}H_{34}O_6$: C, 66.98; H, 8.69
$C_{22}H_{32}O_6$: C, 67.32; H, 8.22
Found: C, 66.68; H, 8.48

Both the methyl ester of this acid and the oxidation product with chromic anhydride of the ester were not obtained as crystals.

Each filtrate in the recrystallizations of the fractions of No.3, No. 4, No. 5, No. 7 and No. 9 which were shown in Scheme 1 was collected and evaporated to dryness and gave 300 mg. of oily gum. The residues were combined with fraction No. 10, esterified with diazomethane and evaporated to dryness. The resulting ester was dissolved in a small amount of ether and the ether solution was poured onto an active alumina (20 g., 200 mesh) column of dimensions 90×20. mm. Only one (fraction 2) out of six elutions (fraction 1 to 6) with 10 ml. of ether each afforded 148 mg. of pale yellow crystal with m.p. 168–170°. The crystal was further recrystallied from methanol and identified as methyl 3,12-dioxo-$\Delta^{4,6}$-choladienate by the mixed melting point determination and infrared comparison.

Analysis Calcd. for $C_{25}H_{34}O_4$: C, 75.34; H, 8.60
Found: C, 75.59; H, 8.65

Succeeding two elutions (fractions 7 & 10) with 30 ml. of ether each afforded 10 mg. of crystals with m.p. 187–189° and about 4 mg. of crystals with m.p. 219–221° respectively. These compounds gave an absorption peak at 241 mμ in alcohol, but were not identifiable. Also one elution (fraction 12) out of two elutions (fraction 11 to 12) with 30 ml. of ethyl acetate each afforded 53 mg. of plates with 175–178°. After further recrystallization from ethyl acetate, the melting point raised to 181–183° and this ester was identified as methyl 7α,12α-dihydroxy-3-oxo-Δ^4-cholenate (*8–9*) by the mixed melting point determination and infrared comparison.

Analysis Calcd. for $C_{25}H_{38}O_5$: C, 71.74; H, 9.15
Found: C; 71.64; H, 9.02

Spectrophotometric Analyses of the Degradation Products of Cholic Acid in S. rubescens Culture—The above-described synthetic medium which was adopted for the isolation of the degradation products of cholic acid was used. 100 ml. of this medium placed in 500 ml. Sakaguchi's flask was autoclaved at 110° for 20 minutes. After inoculation of *S. rubescens*, the inoculated flask was shaken at 27° on a reciprocal shaker (120 r.p.m., 5 cm. amplitude) and an aliquot of the culture medium was separated under aseptic conditions, diluted (1 : 50) and centrifuged at 3.000 r.p.m. for 5 minutes. The ultraviolet absorption spectrum of the supernatant was determined. The relationships between the absorption curves and the incubation periods were shown in Figs 2, 3 and 4.

DISCUSSION

Even when *S. rubescences* is inoculated and incubated in the same condition, there are some differences in the ultraviolet absorption curves of the culture filtrates as seen in Figs. 2, 3 and 4 respectively. An elucidation for such phenomena may be that a degree of the growth of the microorganism probably controls the cholic acid degradation. In Figs. 2 3 and 4, the behaviour of the peaks at 246 and 290 mμ differs slightly from each other.

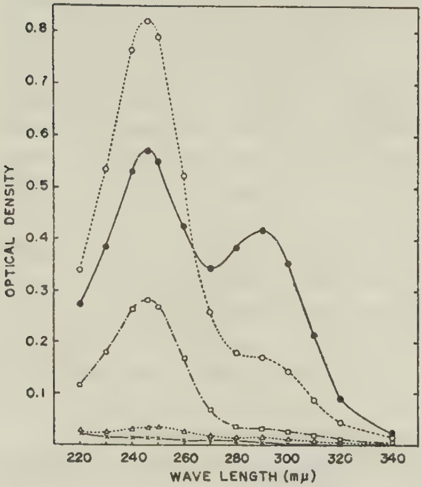


FIG. 2. Ultraviolet absorption spectra of the culture filtrates (diluted 1 : 50) obtained after incubation of cholic acid (0.3 g./dl) with *S. rubescens* at 27° for the following incubation periods: —×—, diluted medium only; ···△···, 2 days; –·–□–·–, 3 days; – –○– – 4 days; —●—, 5 days.

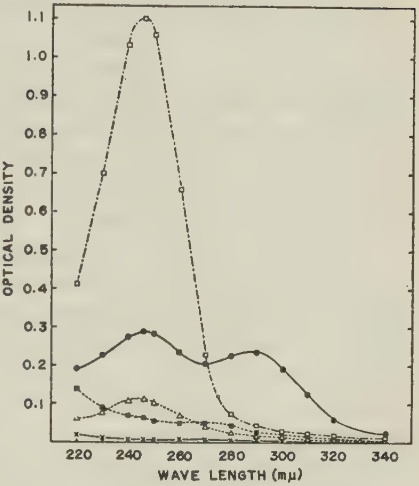


FIG. 3. Ultraviolet absorption spectra of the culture filtrates (diluted 1 : 50) obtained after incubation of cholic acid (0.3 g./dl) with *S. rubescns* at 27° for the following incubation periods: – –■– –, 8 days. See footnotes in Fig. 2.

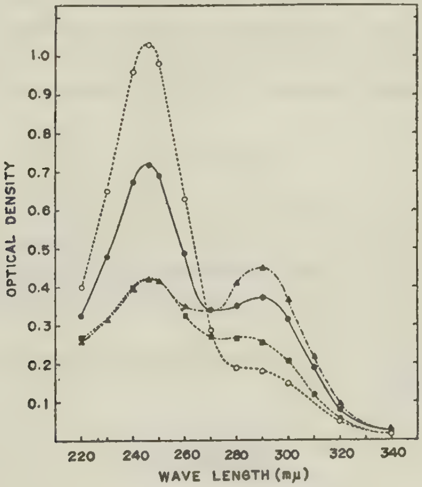


FIG. 4. Ultraviolet absorption spectra of the culture filtrates (diluted 1 : 50) obtained after incubation of cholic acid (0.3 g./dl) with *S. rubescens* at 27° for the following incubation periops: –·–▲–·–, 6 days. See footnotes in Figs. 2 and 3.

However, it is commonly confirmed in those Figs. that *S. rubescens* cultures in a cholate medium give a peak at 246 mμ at the earlier incubation period and a parallelism between the increase of the optical density at 246 mμ and the incubation period is observed. After the optical density at 246 mμ reaches its maximum, it decreases in parallel with the continuation of the incubation and simultaneously, a new peak at 290 mμ appears and finally the both peaks at 246 and 290 mμ decrease or disappear at the end of the incubation period.

In support of the above-proposed pathway in Fig. 1, the observed spectral change may be explained as follows: an appearence of a peak at 246 mμ in the cholate culture begins with the conversions of I→II→III→IV, a decrease of the optical density at 246 mμ and an appearence of a peak at 290 mμ are due to a conversion of IV→V, and further degradation of V accelates a conversion of IV→V and consequently, a decrement or a disappearence of the both peaks at 246 and 290 mμ are observed.

Halperin *et al.* (*10*) have demonstrated that cholic acid is degraded to cholanic acid derivative containing a Δ^4-3-ketone structure by *Nocardia* sp. Further they have isolated two degradation products containing a Δ^4-3-ketone structure and a $\Delta^{4,6}$-3-ketone structure respectively by chromatography using a silica gel column. However, the identification of the compounds is not performed and it is not clear whether the latter compound containing a $\Delta^{4,6}$-3-ketone structure is an intermediate which is produced by enzymatic reaction or it is the artificial transformation product inevitably accompanied by the isolation process.

Therefore the present experiment, succeeded in the isolation and identification of the various intermediates enough to elucidate the data obtained spectrophotometorically, is the first to demonstrate a microbiological introduction of a $\Delta^{4,6}$-3-ketone structure into the cholane nucleus of cholic acid.

SUMMARY

Cholic acid was incubated with *S. rubescens* in a synthetic medium containing this bile acid as the sole source of carbon, and the following compounds were isolated as the degradation products of cholic acid: 7α,12α-dihydroxy-3-oxo-Δ^4-cholenic acid, 7α-hydroxy-3,12-dioxo-Δ^4-cholenic acid, and 3,12-dixo-$\Delta^{4,6}$-choladienic acid.

A possible pathway for the cholic acid degradation by *S. rubescens* was proposed and discussed.

REFERENCES

(*1*) Saburi, Y., Hayakawa, S., Fujii, T., and Akaeda, I., *J. Biochem.*, **43**, 711 (1956)
(*2*) Saburi, Y., Hayakawa, S., and Fujii, T., *Japan. J. Exptel. Med.*, **25**, 151 (1955)
(*3*) Hayakawa, S., *Proc. Japan Acad.*, **30**, 128 (1954)
(*4*) Hayakawa, S., Fujii, T., Saburi, Y., and Eguchi, T., *Nature*, **179**, 537 (1957)
(*5*) Hayakawa, S., Saburi, Y., and Tamaki, K., *Proc. Japan Acad.*, **33**, 221 (1957)

(*6*) Hayakawa, S., Saburi, Y., and Teraoka, H., *Proc. Japan Acad.*, **32**, 519 (1956)
(*7*) Tamaki, K., *J. Biochem.*, in press
(*8*) Eguchi, T., *J. Biochem.*, **44**, 81 (1957)
(*9*) Tamaki, K., *J. Biochem.*, in press
(*10*) Halperin, A. H., Quastel, J. H., and Scholefield, P. G., *Arch. Biochem. Biophys.*, **52**, 5 (1954)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

STUDIES ON PROTAMINES

III. N-TERMINAL RESIDUES OF SALMINE AND CLUPEINE*

BY TOSHIO ANDO, MAKOTO YAMASAKI†, EIKO ABUKUMAGAWA,
SHIN-ICHI ISHII AND YUTAKA NAGAI

(*From the Institute of Science and Technology, University of Tokyo,
and the Department of Chemistry, College of General
Education, University of Tokyo*†, *Tokyo*)

(Received for publication, February 14, 1958)

The results of N-terminal studies with several preparations of protamine sulfates have been briefly reported from our laboratory (*2–4*). Chemical treatment with 1,2,4-fluorodinitrobenzene (FDNB), as well as an enzymatic one with leucine aminopeptidase (*5*) have shown, that proline occupies the N-terminus of salmine from *Oncorhynchus keta*, whereas both alanine and proline occupy those of clupeine from *Clupea pallasii* to various extents. It has also been suggested from the results of reaction of the protamines with nitrous acid, that clupeine has surely an α-amino group while salmine mainly an imino group (*1*).

In this paper, the studies on the N-terminal residues of protamines by chemical treatment are fully described.

EXPERIMENTAL

Material

Protamine Sulfates—As salmine sulfate "the less soluble" one was used, which was prepared from mature milts of salmon (*Oncorhynchus keta*) caught in Hokkaido on October 2, 1950. As for clupeine, the following six specimens were mainly used: "the less soluble" and "the more soluble" clupeine sulfates prepared from herring (*Clupea pallasii*) caught at Mombetsu on May 7, 1947, designated in this paper as clupeine I and clupeine I-1, respectively; "the less soluble", "the more soluble", and "the most soluble" clupeine sulfates come from herring obtained at Yoichi on April 3, 1951 (clupeine II, II-1, and II-2, respectively); "unfractionated" clupeine sulfate prepared from herring caught at Reuke on April 14, 1951 (clupeine III), in this case, clupeine picrate prepared without use of acid, was converted to the sulfate without fractionation in aqueous acetone. Details on the preparation of these protamine sulfates have been described in a previous paper in this series (*6*).

*Preparation of DNP**-Amino Acids and -Peptides*—The DNP-amino acids were prepared by the general method of Sanger (*7*), and Porter and Sanger (*8*). DNP-D, L-Alanine has m.p. 175.5–177.0° (uncorrected). After recrystallization, DNP-L-proline was further purified through Hyflo Super-Cel column to be free from contaminant 2,4-dinitrophenol (*9*). Some DNP-peptides containing arginine* were similarly prepared and purified through talc column (*10*).

---

* For the Paper II, see Ando *et al.* (*1*).

** 2,4-Dinitrophenyl. Customary abbreviations were also used for amino acids appeared in tables.

Preparation of DNP-Protamines—Protamine sulfates were dinitrophenylated by one of the following three methods.

Method 1: By treating with ion-exchange resin, protamine sulfates were converted to the hydrochlorides, which were dinitrophenylated nearly according to the direction of Felix (*11*). A typical example with salmine was as follows. Salmine sulfate (400 mg.) in water (20 ml.) was converted to the hydrochloride by passing through a column (ϕ 1.6 × 26.5 cm., *ca.* 56 ml.) of Amberlite IRA-400 (Cl-form). The column was washed with water until the effluent gave a negative Sakaguchi test**. The combined effluent (*ca.* 75 ml.) was lyophilized and dried over P_2O_5 *in vacuo*. The salmine hydrochloride thus obtained (330 mg.) was dissolved in water (33 ml.). The solution was mixed with $NaHCO_3$ (165 mg.) and FDNB (165 mg.) in ethanol (33 ml.). The mixture was left in a dark place at room temperature (20–25°). After about 20 hours ethanol was removed under reduced pressure, and the deposited FDNB was removed by repeated extraction with ether. A yellow clear solution thus obtained was neutralized to pH *ca.* 5 with 6 *N* H_2SO_4 and then dried under reduced pressure. The dried material was macerated in acetone and left to stand overnight in a cold room. Then the precipitate was repeatedly washed with acetone, then with ether and finally dried *in vacuo*. DNP-Salmine thus obtained (430 mg., including some inorganic salt) was dissolved in water (35 ml.) and adsorbed on a column (ϕ 1.4 × 16 cm.) of Amberlite IRC-50 (XE-64, H-from). After eluting sodium ion by washing the column with *N* acetic acid (*ca.* 20 ml.), the column was further washed with water (*ca.* 180 ml.) until the effluent gave neutral reaction. By this treatment, a considerable part of 2,4-dinitrophenol produced during the dinitrophenylation was also washed down. Finally the DNP-salmine on the column was eluted with 0.15 *N* HCl while cooling the column with ice water, and the acid effluent (*ca.* 120 ml.) was neutralized to pH 4–5 by adding freshly prepared beads of Amberlite IRA-400 (OH-form). In this way, the residual part of dinitrophenol contained in the effluent was completely removed being adsorbed on the resin. After filtering through a glass-filter, the neutral solution was lyophilized to yield DNP-salmine hydrochloride which was dried over P_2O_5 *in vacuo* (yield : 295 mg.).

Method 2: In this procedure the protamine sulfate was directly dinitrophenylated by the usual method of Sanger (*7*). The reaction mixture was acidified with 2 *N* H_2SO_4 and DNP-protamine was precipitated as sulfate by adding absolute ethanol. The precipitate was repeatedly washed with absolute ethanol and then with ether. In our earlier experiments yellow powder of the product thus obtained was used. Besides DNP-protamine sulfate, the product contained a considerable amount of dinitrophenol and Na_2SO_4. In our later experiments, however, this DNP-product was further purified by Method 1 to be free of such contaminants, and the DNP-protamine hydrochloride thus obtained was used.

---

* Glycyl-L-arginine, D,L-alanyl-L-arginine, L-prolyl-L-arginine, and L-leucyl-L-arginine were supplied by Dr. T. Tobita in our laboratory, who prepared them to use for other purposes.

** When the protamine was washed out, SO_4^{--} on the column was eluted with *N* HCl. After concentration, SO_4^{--} in the effluent was precipitated and weighed as $BaSO_4$. The contents of H_2SO_4 thus determined were 19.4 per cent and 18.7 per cent for salmine sulfate stored over $CaCl_2$ at room temperature in an atmospheric pressure, and 20.6 per cent and 20.4 per cent for clupeine sulfate II dried to constant weight at 110° *in vacuo* over

Method 3: The protamine sulfate in water was treated with $BaCl_2$ to yield the hydrochloride and this was dinitrophenylated in the presence of $BaSO_4$. An example with clupeine was as follows. The clupeine sulfate (10 mg.) in water (0.5 ml.) contained in a tube with glass stopper was converted to the hydrochloride by adding 0.1 *M* $BaCl_2$ (0.25 ml.). The solution was mixed with $NaHCO_3$ (10 mg.) and FDNB (7–8 mg.) in ethanol (0.7 ml.). The mixture was allowed to stand in a dark place at 30°. After about 20 hours, 6 *N* HCl (3 drops) was added and the acid solution (pH *ca.* 1.6) was extracted with ether (4 ml.). After centrifugation the ether layer was removed. By repeating the extraction with ether four times more, the FDNB remained unreacted and the dinitrophenol produced during the dinitrophenylation were thoroughly removed. The aqueous layer was pipetted and filtered through a small glass-filter without suction. $BaSO_4$ remained in the tube was washed four times with water (1 ml. each) and after centrifugation the clear washings were filtered. The combined filtrate was neutralized with 2 *N* NaOH to pH 4–5 and was dried *in vacuo* in a desiccator over solid NaOH and conc. H_2SO_4. The DNP-protamine hydrochloride thus obtained contained a considerable amount of NaCl but was used as it is for the purpose of absorption measurement and acid hydrolysis.

Analytical Methods

Measurements of Absorption Spectra—For the measurement of absorption spectra of DNP-derivatives, a Beckman photoelectric spectrophotometer, model DU, with 1 cm. silica cuvettes was used. 1 *N* (or 0.1 *N* in some cases) HCl, 1 per cent $NaHCO_3$, and 0.25 *N* acetic acid were used as solvent. In every case, the concentration of the DNP-group was less than 50 μmole per liter to conform to Beer's law.

Chromatographic Separation and Determination of DNP-Derivatives—Ether-soluble DNP-derivatives were separated by the column chromatography of Perrone (*9*) using buffered Hyflo Super-Cel* (Johns-Manvill Co.) instead of Celite 543. The columns (*ca.* 0.9 cm. in diameter) consisted of two layers; the upper layer (*ca.* 3.5 cm.) was buffered with *M* phosphate buffer of pH 5.4 and the lower (*ca.* 5 cm.) with 0.4 *M* one of pH 6.5. Both layers were buffered with 0.6 ml. of the buffer per 1 g. of the Hyflo Super-Cel. When developed with 4–5 ml. of ether saturated with water, statisfactory separation resulted. Each band containing DNP-groups was cut out, eluted with 1 per cent $NaHCO_3$, and determined colorimetrically. For the accurate determination of dinitrophenol, this column chromatography was found to be particularly useful.

Ether-soluble DNP-derivatives were also identified and estimated by the two-dimensional paper chromatography of Levy (*12*), using the Toyo No. 51 filter paper. For the detection and estimation of minute components, this procedure was found especially suitable.

Acid-soluble DNP-derivatives were chromatographed by running in *tert*-amyl alcohol saturated with 0.1 *M* phthalate buffer (pH 5.4) on the similarly buffered No. 51 paper (*13*). In our case, the acid soluble fractions often contained arginine, which was revealed by the positive Sakaguchi test on paper (*14*).

In our earlier experiments, DNP-derivatives were chromatographed on the Toyo No. 50 filter paper using phenol system (*15*), toluol system (*15*), and butanol-acetic acid system as solvent. The composition of these solvents are given under Table I.

Paper Electrophoresis—Paper electrophoresis was carried out using a B type apparatus of Natsume Seisakushyo Ltd. The Toyo No. 51 filter paper and 0.25 *N* acetic acid (pH 2.8) were used throughout. The over-all length was 23 cm. between the fluid levels, and the horizontal portion was of 16 cm. length.

---

* The authors are grateful to Prof. K. Satake of Tokyo Metropolitan University for his kind supply of this Hyflo Super-Cel preparation.

Assay of Proline—To identify proline on a paper chromatogram, the isatin test was used (*17*). Proline was determined colorimetrically by the ninhydrin procedure of Chinard (*18*).

RESULTS

Dinitrophenylation of Protamines

In Methods 1 and 3, the mixture was homogeneous during the dinitrophenylation reaction, while in Method 2 the reaction proceeded in a heterogeneous medium, since the protamine sulfate is rather difficultly soluble in aqueous ethanol and precipitates soon after the reaction started. In Method 2, therefore, it was observed that the yield of DNP-product from a small amount of protamine sulfate (less than 50 mg.) was considerably affected by small differences in the reaction conditions.

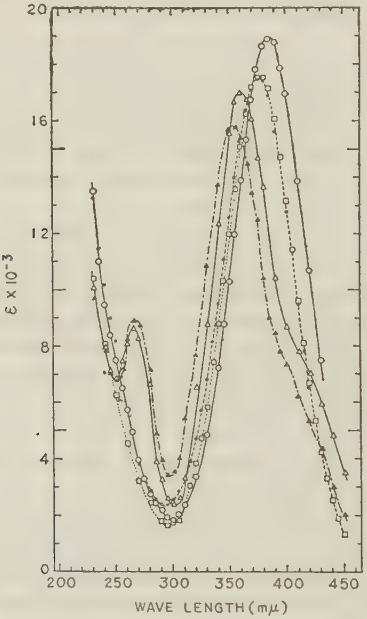


FIG. 1. Absorption spectra of DNP-L-proline, DNP-L-prolyl-L-arginine and DNP-D,L-alanine in 1 per cent sodium bicarbonate and *N* hydrochlolic acid. DNP-Proline in 1 per cent $NaHCO_3$ (—○—) and in *N* HCl (--●--). DNP-Prolylarginine in *N* HCl (···□···). DNP-Alanine in 1 per cent $NaHCO_3$ (—△—) and in *N* HCl (·—·▲—·).

Method 3 was very convenient, and even a small quantity less than 10 mg. of the protamine sulfate was successfully dinitrophenylated. The product obtained by this procedure contained a considerable amount of sodium chloride, but was found to be useful in this state for the measurement of absorption spectrum and for acid hydrolysis. Almost no difference was found between the results obtained with the DNP-products prepared by this method and by the other two, where in the latter, the DNP-protamine was prepared in a larger scale and was free from inorganic salt.

In every procedure mentioned above, a careful investigation of the organic solvent used for washing the DNP-protamine, showed no presence of DNP-amino acid or -peptide.

Absorption Spectra of DNP-Protamines

Fig. 1 shows the absorption spectra in the region of 450–230 mμ of DNP-proline, DNP-prolylarginine and DNP-alanine in 1 per cent sodium bicarbonate and N hydrochloric acid. The results of our earlier experiments are also summarized in Table I.

Table I

R_f Values and UV-Absorption Maxima of Some DNP-Derivatives (Earlier Experiment)*

	Phenol system	Toluol system	BuOH-AcOH system	Absorption maxima** (mμ)	
				λ_1 max	λ_2 max
DNP-Arg	0.80	0.55	0.60	*ca.* 362	265
DNP-Ileu	0.68		0.93	362	265
DNP-Val	0.67	0.54	0.90	363	265
DNP-Pro	0.51	0.39(0.37§)		*ca.* 387	none
DNP-Ala	0.46	0.43(0.37§)	0.80	361	265
DNP-Gly	0.32	0.33	0.67	361	265
DNP-Ser	0.18	0.19	0.67	359	265
DNP-OH†	0.21	0.32	0.80	361	257
DNP-NH_2††	0.91		0.82	347	262

* The Toyo No. 50 filter paper was used throughout. Results by descending chromatography at room temperature (20°). Phenol system (*15*): Top layer of the mixture, phenol: *iso*-amyl alcohol: $H_2O=1:1:1$ (*w/w*). Toluol system (*15*): Toluol: pyridine: ethylenechlorohydrin: 0.8 N aq. ammonia $=5:1:3:0.3$ (*v/v*). In the above two solvent systems, directions of Biserte and Osteux (*15*) were referred to. BuOH-AcOH system: *n*-Butanol: acetic acid: $H_2O=4:1:1$ (*v/v*).

** Measured in 1 per cent $NaHCO_3$. Details have already been published by Hashimoto (*16*) of this laboratory.

§ R_f value in the presence of DNP-OH.

† 2,4-Dinitrophenol.

†† 2,4-Dinitroaniline.

Figs. 2 and 3 show the absorption spectra of DNP-protamines in 1 per cent sodium bicarbonate and N hydrochloric acid. In these figures, the value of the molecular extinction coefficient (ε) of DNP-protamines at 360 mμ is taken for 1.54×10^4 on the basis to be described under the discussion part,

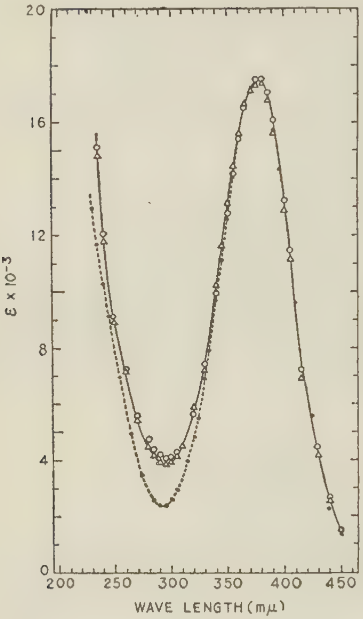


FIG. 2. Absorption spectra of DNP-salmine in 1 per cent sodium bicarbonate and *N* hydrochloric acid. DNP-Salmine in 1 per cent $NaHCO_3$ (—△—) and in *N* HCl (—○—). Absorption spectrum of DNP-L-proline in *N* HCl (- - ● - -) is shown for comparison.

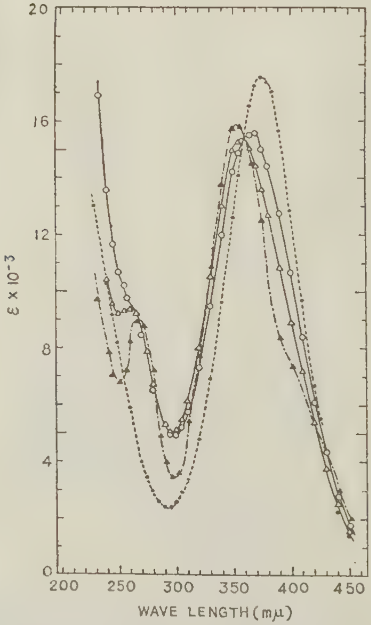


FIG. 3. Absorption spectra of DNP-clupeine II and DNP-clupeine III in *N* hydrochloric acid. DNP-Clupeine II (—○—) and III (—△—). Absorption spectra of DNP-L-proline (- - ● - -) and DNP-D,L-alanine (·—·▲—·) in *N* HCl are shown for comparison.

and the curves of DNP-protamines are compared with those of DNP-proline or DNP-alanine in *N* hydrochloric acid.

The ratio (designated as R) of an optical density at 390 mμ to that at 360 mμ has a characteristic value for absorption curves of DNP-amino acids and -peptides (*19*). Therefore the R value found with various DNP-derivatives in 1 per cent sodium bicarbonate and/or N hydrochloric acid are shown in Table II.

TABLE II

The Values of R† and the Calculated Percentage of N-Terminal Proline in Protamines

DNP-Derivatives	R (λ_1 max, mμ)		Percentage of N-terminal proline calculated from R
	in 1% $NaHCO_3$	in N HCl	
DNP-Ala	0.60–0.62 (362)	0.55 (353)	
DNP-Pro	1.36 (385)	1.02 (375)	
DNP-Gly		0.54 (*ca.* 352)	
DNP-Thr		0.54* (*ca.* 352)	
DNP-Ala·Gly·Gly	0.54** (*ca.* 353)		
DNP-Gly·Gly·Gly	0.56*** (352)		
DNP-OH	0.81	0.12	
DNP-Clupeine I§	0.69 (*ca.* 357)	0.67 (*ca.* 357)	25
II§§	0.83 (*ca.* 365)	0.83 (*ca.* 365)	59
II§§		0.78 (360–363)	49
II		0.78 (360–363)	49
II-1		0.74 (360)	40
II-2		0.66 (353–356)	23
III		0.72 (358–362)	36
DNP-Salmine§	1.00 (375)	1.00–1.04 (375)	100

† R represents the ratio of optical densities at 390/360 mμ.

* Calculated from the absorption spectrum in 0.1 N HCl given by Phillips (*20*).

** Calculated from the absorption spectrum given by Levy *et al.* (*21*).

*** Calculated from the absorption spectrum given by Hashimoto (*16*).

§ Dinitrophenylated by Method 1.

§§ Dinitrophenylated by Method 2; other specimens of DNP-clupeine were dinitrophenylated by Method 3.

Fig. 4 shows a part of the results obtained by the measurements of absoption curves in N hydrochloric acid of the mixture of DNP-proline and DNP-alanine in various proportions. The relation between compositions of mixtures and their R values is indicated in Fig. 5.

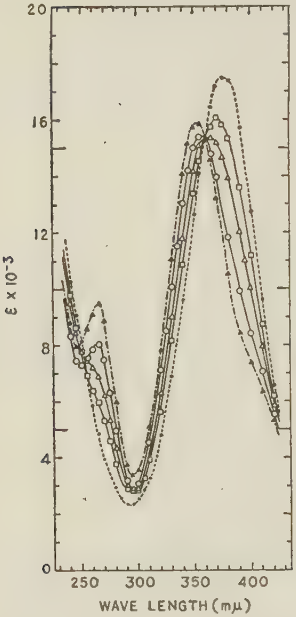


FIG. 4. Absorption spectra of mixtures containing various molar fractions of DNP-D,L-alanine and DNP-L-proline in N hydrochloric acid. Molar ratios of DNP-alanine: DNP-proline, 1.0 : 0.0 (·—·▲—·), 0.8 : 0.2 (—○—), 0.5 : 0.5 (—△—), 0.3 : 0.7 (—□—), and 0.0 : 1.0 (--●--).

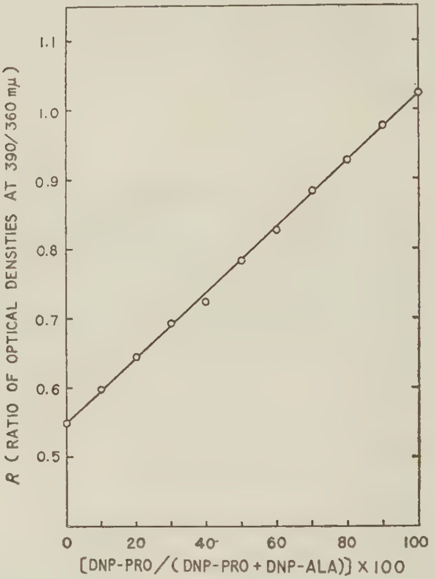


FIG. 5. The relation between the composition of the mixture (DNP-D,L-alanine+DNP-L-proline) and the ratio of optical densities at 390/360 mμ of absorption curves of the mixture measured in N hydrochloric acid.

Identification and Determination of N-Terminal Residues by Acid Hydrolysis of DNP-Protamines

a. Identification of Ether-Soluble and Acid-Soluble DNP-Derivatives Obtained from the Hydrolysates (*Earlier Experiments*)—Table I shows the R_f values of paper chromatography in various solvent systems and the absorption maxima in 1 per cent sodium bicarbonate solution of the DNP-derivatives of amino acids found in protamines and also some DNP-compounds to be produced on acid hydrolysis of some DNP-derivatives.

Table III shows the identification of the ether-soluble and acid-soluble DNP-derivatives obtained from the acid hydrolysates of various specimens of DNP-protamine sulfates. The acid hydrolysis was made in a sealed tube using 11.4 N or 5.7 N hydrochloric acid at 107° for 8–16 hours. The result with similarly treated DNP-proline is also shown in the table for comparison, as the compound was found to be very labile to acid hydrolysis.

b. Regeneration of the N-Terminal Amino Acid by Decomposition with Ammonia of DNP-Derivatives Obtained in the Acid Hydrolysates of DNP-Clupeine and -Salmine

TABLE III

R_f Values and Absorption Maxima of DNP-Products Obtained in the Acid Hydrolysates of DNP-Protamines (Earlier Experiment)***

DNP-Derivatives hydrolyzed	Hydrolysis		R_f values in			UV-Absorption maxima		Assignment
	agent (concn. of HCl)	time (*hr.*)	phenol system	toluol system	BuOH-AcOH system	λ_1 max ($m\mu$)	λ_2 max ($m\mu$)	
DNP-Clupeine I						357	262	***
Ether fraction	5·7 *N* 11·4 *N*	8 16	0.21 0.47	0.32 0.36				DNP-OH DNP-Ala
Aq. fraction§	5.7 *N*	8	0.21					?
	11.4 *N*	16	0.21 0.62					? ?
DNP-Clupeine I-1								
Ether fraction	11.4 *N*	10	0.21 0.47	0.32 0.37				DNP-OH DNP-Ala
Aq. fraction§§	,,	,,	0.93 0.71 0.25	0.92 0.50 0.32	0.77 0.65 0.42			DNP-NH_2? ? ?
DNP-Clupeine III						360	(?)	***
Ether fraction	11.4 *N*	16	0.17 0.37 (0.47∼)	0.32 0.33				DNP-OH DNP-Ala (?)
Aq. fraction	,,	,,		0.16				?
DNP-Clupeine†						359	(?)	***
Ether fraction	11.4 *N*	16	0.17 0.38 (0.47∼)					DNP-OH DNP-Ala (?)
Aq. fraction	,,	,,		0.14				?
DNP-Salmine						375	none	DNP-Prolyl peptide
Ether fraction	11.4 *N*	16	0.21 0.62	0.31 0.50				DNP-OH Deriv. from DNP-Pro
Aq. fraction§	,,	,,	0.21					?
DNP-Proline								
Ether fraction	11.4 *N*	16	0.21 0.61	0.30 0.53				DNP-OH Deriv. from DNP-Pro
	,, ,,	10 1	0.21 0.50 0.61	0.30 0.50 0.60				DNP-OH Deriv. from DNP-Pro
	5.7 *N*	8	0.21	0.30				DNP-OH
Aq. fraction			—	—	—			(Only free Pro by ninhydrin)

* The conditions for paper chromatography were the same as in Table I, except for the hydrolysates of DNP-clupeine III and DNP-clupeine†. In the latter cases, R_f values of DNP-OH and DNP-Ala for control were found to be 0.17 and 0.38 in phenol system, and 0.31 and 0.33 in toluol system, respectively.

** UV-Absorption measurement and some paper chromatographic identification were kindly made by Dr. C. Hashimoto of this laboratory.

*** Supposed at that time to be only DNP-amino acid other than DNP-Pro in the N-terminal.

§ After extraction with ether, the acid solution was extracted with methylethyl ketone: ether=2: 1 (*v/v*), and this extract was examined. No spot corresponding to DNP-Arg was found.

§§ Methanolic extract of the acid solution was used, accompanying free amino acids being removed as possible by precipitation with the addition of methylethyl ketone to the extract. There appeared several yellow spots which seemed to be DNP-peptides due to shorter time hydrolysis. No spot of DNP-Arg was found.

† "Unfractionated" clupeine sulfate prepared from the same milts as for clupeine III was used, but the picrate was prepared, as usual, through the extraction with diluted HCl.

(*Earlier Experiments*)—The ether-soluble DNP-derivatives (4.5 mg. in each case) obtained from the hydrolysates of DNP-salmine and DNP-clupeine I were heated with 33 per cent ammonia (1 ml. each) in sealed tubes for 4 hours at 100° (*22*). After the removal of ammonia the decomposition products were identified on one-dimensional paper chromatogram using *n*-butanol-acetic acid-water (4 : 1 : 1, *v/v*) and 80 per cent aqueous phenol as developing solvents. The only amino acid detected in the product from DNP-clupeine I was alanine, while no significant spot was found in that from DNP-salmine.

c. Identification and Determination of Ether-Soluble DNP-Derivatives—DNP-Protamine hydrochlorides (6–8 mg.) were hydrolyzed in sealed tubes with 6 *N* hydrochloric acid (0.4–0.5 ml., redistilled in all-glassware) at 100° for 2, 6, 8 and 12 hours. Each hydrolysate was diluted with an equal volume of water and extracted repeatedly with ether (4 ml. × 5). The combined ether extracts were washed with 0.1 *N* hydrochloric acid (1 ml.) and ether was evaporated under a stream of air at 30–35°. The ether-soluble DNP-derivatives thus obtained were separated by chromatography on a buffered Hyflo Super-Cel column (*9*) or by two-dimensional paper chromatography according to Levy (*12*). The DNP-derivatives thus separated and identified were eluted with 1 per cent aqueous sodium bicarbonate and determined colorimetrically. When, prior to the chromatography, dinitrophenol was necessary to be removed from the ether-soluble DNP-derivatives, it was sublimed *in vacuo* at 60° (*23*).

For comparison, DNP-proline and DNP-alanine were similarly treated (*ca.* 1 μmole of DNP-amino acid in 0.4–0.5 ml. of 6 *N* hydrochloric acid) and their stability to acid hydrolysis and the hydrolytic products were investigated. In the case of DNP-proline which was found to be very labile to acid hydrolysis, the aqueous phase of the hydrolysate after the ether extraction was once evaporated to dryness. The dried matter was dissolved in water, and the proline regenerated in the solution was determined colorimetrically (*18*) and identified by one-dimensional paper chromatography using *n*-butanol-acetic acid-water (4 : 1 : 1, *v/v*) as solvent. When revealed with ninhydrin and isatin (*17*), only proline was found as a colored spot. The results

TABLE IV

Amounts of Compounds Obtained by the Acid Hydrolysis of DNP-Proline

Time of hydrolysis (*hr.*)		2		6	8		24
Salmine sulfate*		Not added	Added	Not added	Not added	Added	Not added
Ether-soluble DNP-compounds recovered (*mole/mole*)	DNP-Pro	0.05	0.07	0	0	0	0
	DNP-OH**	0.88	0.85	0.82	0.88	0.86	0.93
Pro regenerated during acid hydrolysis (*mole/mole*)***		0.76	—	1.1_2	1.0_6	—	1.1_1

In every case, 0.76 μmole each of DNP-Pro was hydrolyzed with 0.4 ml. of 6 *N* HCl at 100° in a sealed tube. Separation and estimation of the ether-soluble compounds were made with the Hyflo Super-Cel column, and the chromatographic loss, estimated to be several per cents at most, was not corrected.

* As control, 6 mg. of salmine sulfate was added in some cases.

** ε of DNP-OH at 360 mμ in 1 per cent $NaHCO_3$ was taken for 1.48×10^4.

*** Pro was determined according to the method of Chinard (*18*) by Dr. M. Sato of this laboratory, to whom the anthors' thanks are due.

TABLE V

Recovery of DNP-Alanine after Its Acid Hydrolysis

Time of hydrolysis (*hr.*)	6	8		12
Clupeine hydrochloride*	Added	Not added	Added	Added
DNP-Ala recovered (*mole/mole*)	0.96	0.98	0.96	0.94

Separation and estimation of DNP-Ala were made with Hyflo Super-Cel column, and the chromatographic loss of 6 per cent (obtained from control experiments) was corrected. In every case, 1.18 μmole of DNP-Ala was hydrolyzed with 0.4 ml. of 6 *N* HCl at 100° in a sealed tube.

* As control, 6 mg. of clupeine hydrochloride was added to each tube.

obtained in the above are summarized in Tables IV–VI.

d. Purification and Identification of Acid-Soluble DNP-Derivatives—In our earlier experiments, investigation of the acid-soluble fraction revealed no DNP-arginine (see Table III). In the recent study, however, the results obtained were somewhat different.

After the extraction with ether, the aqueous layer of the hydrolysate and the washing solution with 0.1 *N* hydrochloric acid were combined and dried under reduced pressure. The residue was dissolved in hydrochloric acid and its absorption was read in the region of 450–300 mμ. Fig. 6 shows typical absorption spectra of these residues in hydrochloric acid. It is clear from the figure that the curves lose the characteristics of DNP-peptides as the hydrolysis proceeds. The last line of Table VI shows percentages of the absorption of the residues in hydrochloric acid to that of the original

TABLE VI

Amounts of DNP-Derivatives Recovered after Acid Hydrolysis of DNP-Protamines

Protamine		Salmine*			Clup*. I	Clupeine** II				Clup.** II	Clup. II	Clup. II-1	Clup. II-2	Clup. III
Chromatographic method***		CC		PC	CC	CC				PC	PC	PC	PC	PC
Time of hydrolysis (*hr.*)		2	8		8	6	8		12	8	8	8	8	8
Mole of N-terminal alanine per mole of DNP-protamine estimated from *R* value		0			0.75	0.41				0.51	0.51	0.60	0.77	0.64
Mole of ether-soluble DNP-derivatives recovered per mole of DNP-group after hydrolysis****	DNP-Ala				0.70	0.41	0.39	0.41	0.34	0.45	0.42	$\left\{\begin{matrix}0.41\\0.49\end{matrix}\right.$	$\left\{\begin{matrix}0.54\\0.57\end{matrix}\right.$	$\left\{\begin{matrix}0.44\\0.52\end{matrix}\right.$
	DNP-OH	0.62	0.69	+		§	§	0.33	§	+	+	+	+	+
	DNP-Pro	0.03	0	0.0_6						(+)	(±)	(±)		(±)
	DNP-Ser			(+)						(±)	(±)	(±)	(±)	(+)
	Spot 1†										(±)			(+)
	Spot 2††												(+)	
Amount (%) of DNP-derivatives remained in acid†††		23	11	10	9	16	11	11	5	10	7	6	6	6

* Dinitrophenylated by Method 1.

** Dinitrophenylated by Method 2; others were by Method 3.

*** CC: Separated by Hyflo Super-Cel column; in this case, chromatographic loss of 6 per cent was corrected only for DNP-Ala obtained.

PC: Separated by two-dimensional paper chromatography (Levy's method); in this case, chromatographic loss of 10 per cent was corrected for each of the ether-soluble derivatives obtained.

**** Correction for the destruction by acid hydrolysis was applied only to DNP-Ala using the factors in Table V.

† An unidentified spot, which located near DNP-Val on the two-dimensional paper chromatogram, but had a λ_{max} at *ca.* 430 mμ and might not be a DNP-amino acid.

†† An unidentified spot, located near DNP-Phe.

††† Calculated from the absorption at 375 mμ for DNP-salmine and at 360 mμ for DNP-clupeine.

§ Considerable amount of DNP-OH was found but not determined.

+ A distinct yellow spot was found on the paper chromatogram.

(+) A perceptible faint yellow spot was found on the paper chromatogram, but its amount did not exceed 0.01 mole per mole of DNP-group.

(±) A scarcely perceptible yellow spot, less than (+) in amount.

DNP-groups hydrolyzed, when compared at 375 mμ for salmine and at 360 mμ for clupeine.

Each amount of absorption in acid solution that remained after 8 hour hydrolysis was less than 11 per cent of that of the original DNP-groups (*cf.* Table VI). After the measurement of absorption, these residual DNP-derivatives were purified for further investigation by passing the solution through talc* column (ϕ 1.5×1.5 cm.) (*10*) and washing with *N* hydrochloric acid (30 ml.). Free peptides and amino acids passed rapidly down the column, while the yellow material ("acid-soluble DNP-derivatives") was retained in a band at the top of the column. The yellow band was rapidly eluted with a mixture of four parts of ethanol and one part of *N* hydrochloric acid. The effluent was dried under reduced pressure. A part of the acid-soluble DNP-derivatives thus obtained was examined by paper electrophoresis in 0.25 *N* acetic acid and the remainder by paper chromatography using *tert*-amyl alcohol-phthalate system (*13*). The results with DNP-salmine and DNP-clupeine II are shown in Figs. 7 and 8 as typical

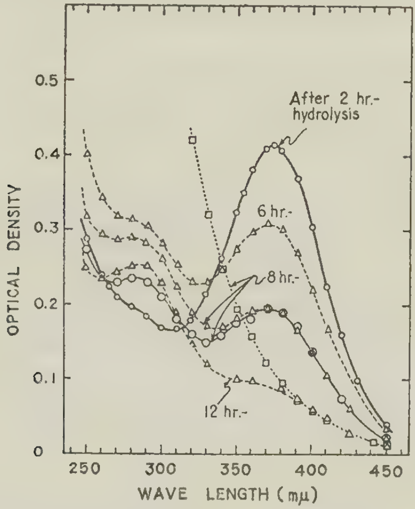


FIG. 6. Absorption curves of DNP-derivatives remained in the acid phase after extraction with ether of acid hydrolysates of DNP-salmine and DNP-clupeine. Hydrolysis was made in a sealed tube with 6 *N* HCl at 100°. The amount of absorption of each acid-soluble substance was measured in 10 ml. of *N* HCl solution. 1.05 μmole each of DNP-salmine (—○—) prepared by Method 1 in the text was used for hydrolysis; 1.17 μmole each of DNP-clupeine II (‐‐△‐‐) by Method 2; 1.77 μmole of DNP-clupeine III (···□···) by Method 3.

---

* A preparation of Kozakai Siyaku Co. Ltd. Before use the talc was washed several times by decantation with *N* HCl at 60°.

examples. In the case of salmine, the acid-soluble DNP-derivatives separated into two spots of about the same size in either procedure. A set of corresponding spots (A and A_1) was similar in behavior to DNP-arginine and showed a positive Sakaguchi test on paper (*14*). Another set (B and B_1) was similar in that each one gave an abnormal Sakaguchi reaction, *i.e.* it turned purple when sprayed with α-naphthol solution and decolored on spraying with sodium hypobromite solution, and it may probably be an identical but unidentifiable material. As to the case of clupeine, the paper electrophoretic study revealed two yellow spots showing the positive Sakaguchi test, one (C) with the behavior similar to DNP-arginine and another (D) with larger mobility and richer in amount. The latter spot (D) from clupeine II was eluted with N hydrochloric acid and the absorption curve was determined. It had an absorption maximum at 375 mμ and the R value was found to be

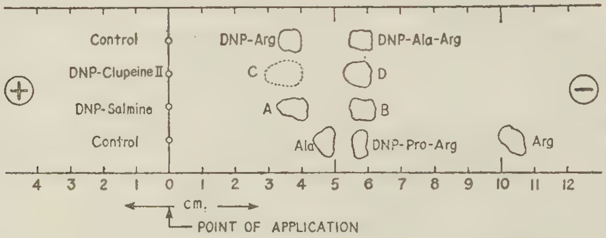


FIG. 7. Pattern obtained following filter paper electrophoresis of acid-soluble DNP-derivatives resulted from acid hydrolysis of DNP-protamines. Electrophoresis was performed for 45 minutes on the Toyo No. 51 filter paper in 0.25 N acetic acid, applying 0.4 mA/cm. and 20 V/cm. Patterns of DNP-arginine, DNP-alanylarginine, DNP-prolylarginine, alanine and arginine are also shown for control.

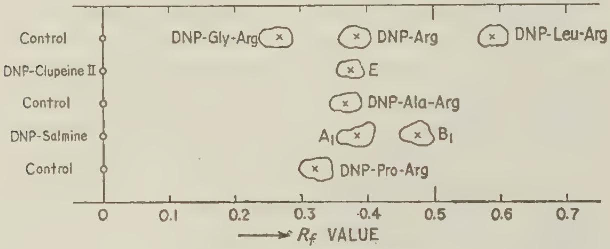


FIG. 8. Pattern of paper chromatography of acid-soluble DNP-derivatives obtained from acid hydrolysates of DNP-protamines. Descending paper chromatography was carried out, using the solvent system of *tert*-amyl alcohol-0.1 M phthalate buffer (pH 5.4), on the similarly buffered No. 51 paper. R_f values were found somewhat to fluctuate in each chromatogram. Patterns of DNP-arginine, DNP-glycylarginine, DNP-alanylarginine, DNP-prolylarginine and DNP-leucylarginine are also shown for control.

1.0. The spot (E) on the paper chromatogram gave the positive Sakaguchi reaction. From the control experiment, it has also been found that the DNP-derivatives of arginine-containing peptides, such as alanylarginine and prolylarginine, behaved very similar to DNP-arginine on this paper chromatogram. Hence the identification of DNP-arginine in this case was found rather obscure.

DISCUSSION

As shown in the previous paper (*6*), both the salmine and the clupeine have no absorption band in the region of wave length longer than 250 mμ and contain only an α-amino or -imino group but no such residues as lysine, tyrosine, histidine and cysteine to cause absorption band in the near ultraviolet region in reaction with FDNB under usual conditions.

It was found by Hashimoto (*16*) in this laboratory, that the DNP-derivatives of the constituent amino acids of protamines (arginine, alanine serine, valine, threonine, glycine, isoleucine and proline for clupeine and those except threonine for salmine) could be classified in two types from their way of absorption in the near ultraviolet region in 1 per cent sodium bicarbonate solution. One is a DNP-proline type showing only one absorption maximum at 385 mμ, and another an α-DNP-amino acid type other than proline, showing maxima at about 360 ($\lambda_{1\,\max}$) and 265 mμ ($\lambda_{2\,\max}$), as represented by DNP-alanine (see Fig. 1 and Table I). Based on these results, Hashimoto attempted to identify the N-terminal residues from the absorption curve of DNP-protamines (*16*). A quite similar attempt was independently made on DNP-salmine by Phillips (*20*). Since such a procedure needs no acid hydrolysis, it has proved to be particularly useful in the case of salmine having proline in the N-terminus, the DNP-derivative of which always suffers an extensive decomposition on its hydrolysis.

As solvents for absorption measurement, aqueous sodium bicarbonate, hydrochloric acid or glacial acetic acid (*24*) have been commonly used and the absorption spectra of DNP-amino acids are affected considerably by the solvents used. As seen in Fig. 1, DNP-proline and DNP-alanine in *N* hydrochloric acid have both the absorption curves in which the $\lambda_{1\,\max}$ was shifted by *ca.* 10 mμ to the shorter wave length region and the molecular extinction coefficient (ε) at the $\lambda_{1\,\max}$ lowered by *ca.* 10 per cent, when compared to those in 1 per cent sodium bicarbonate. The results obtained by Phillips (*20*) with DNP-proline and its methyl ester suggested, that the ε value of an α-DNP-amino acid in hydrochloric acid (0.1–1 *N*)* could be used as that of the α-DNP-group in the DNP-peptide. This fact was also verified in our experiments. We have found, that the absorption curve of DNP-proline in *N* hydrochloric acid showed a fine agreement with that of DNP-prolylarginine in this solvent (see Fig. 1). It has also been found, that in the peptide form, the absorption spectra were little affected by either solvent used. Thus DNP-prolylarginine gave almost the same spectra either in *N* hydrochloric acid or in 1 per cent sodium bicarbonate. Similarly, the absorption curve of DNP-alanine in *N* hydrochloric acid is in good accord with that* of DNP-alanylglycylglycine in 1 per cent sodium bicarbonate. Furthermore, when a mixture of DNP-di- and -tripeptides were obtained by the sufficient digestion of DNP-salmine and -clupeine with trypsin, nearly no change could be found in the absorption (maximum and intensity) between 450–300 mμ before and after the digestion. Thus in our case of protamines, it was shown, that the length of the following peptide chain almost has no effect on the absorption of the DNP-group. In the present paper, therefore, the $\varepsilon_{375\,m\mu}$ value ($1.7_5\times10^4$) of DNP-proline in *N* hydrochloric acid was used in calculation for that of DNP-prolyl peptides, and the $\varepsilon_{355\,m\mu}$ ($1.5_8\times10^4$) of DNP-alanine for that of DNP-alanyl peptides. In the case of a mixture of DNP-prolyl and DNP-alanyl peptides, just as in our DNP-clupeine, the $\varepsilon_{360\,m\mu}$ value ($1.5_4\times10^4$) at the crossing of both curves was used for that of the DNP-peptides. Also $\varepsilon_{385\,m\mu}$ ($1.9_0\times10^4$) and $\varepsilon_{360\,m\mu}$ ($1.7_0\times10^4$) were employed respectively, as those values of DNP-proline and DNP-alanine in 1 per cent sodium bicarbonate.

---

* 0.1 *N* HCl was used by Phillips (*20*). In our case, however, DNP-alanine and DNP-clupeine gave the same absorption curves, respectively, either in 0.1 or 1 *N* HCl.

Using the ε values shown in the above, the molecular weight of the protamines could be calculated to be in a region of 6,000±400 as sulfate. These values of molecular weight are quite in accord with those obtained by other methods which were described in the preceding paper (*1*). Thus it can be considered that the dinitrophenylation of N-terminal of the protamines has proceeded nearly quantitatively. From the results of dinitrophenylation of salmine under conditions considerably similar to our Method 3, Phillips has also shown that the reaction was considered to be completed after 4.5 hours (*20*). Accordingly, we will make our further discussion, supposing the dinitrophenylation of our protamines to be complete.

It can at first be pointed out, that almost no difference could be found between the absorption curves of DNP-protamine either in 1 per cent sodium bicarbonate or in *N* hydrochloric acid, as evidently seen from an example of the DNP-salmine in Fig. 2. Though not shown in this figure, nearly the same curve could also be obtained for the DNP-protamine in 0.25 *N* acetic acid.

Extending over a wide range of wave length, the absorption curve of DNP-salmine** is in fine accord with that of DNP-prolyl type peptides, thus suggesting the N-terminal residue to consist exclusively of proline (see

---

* Given by Levy *et al.* (*21*) in their description.

** Phillips has determined the absorption of DNP-salmine in 0.1 *N* HCl (*20*). Since his specimen of salmine contained together with proline, also other N-terminal amino groups in an amount of *ca.* 20 per cent in total, which should be considered to be contaminated due to the impurity of the specimen or to some decomposition product of salmine during the preparation, the absorption curve of the specimen could hardly agree with that of the DNP-proline for control.

Fig. 2.). This suggestion is quite consistent with the findings by many researchers on salmine from European and American sources (*8*, *11*, *25–27*). On hydrolysis with 6 *N* hydrochloric acid, only a slight quantity of DNP-proline (0.03–0.06 mole/mole DNP-group) was recovered from our DNP-salmine and the most part of DNP-group was obtained in the form of dinitrophenol (at maximum, 0.7 mole/mole DNP-group) (see Table VI). This result agrees with that of the control experiments on DNP-proline as shown in Table IV. It has been shown, that, on hydrolysis with 6 *N* hydrochloric acid, DNP-proline was rapidly decomposed, while dinitrophenol and free proline were obtained almost quantitatively. Thus the result on the stability of the DNP-proline also agrees with the facts found by other investigators (*8*, *28–30*).

As to clupeine, the phenomena are not so simple. The absorption curves of the DNP-clupeine near the ultraviolet region are shifted from that of the DNP-alanyl type towards that of the DNP-prolyl one to various extents as the specimens of clupeine differ (Fig. 3)*. This suggests that both the amino and imino groups in various ratios sit at the N-end of our specimens of clupeine. The changes in the absorption curve of the DNP-alanyl type peptide mixed with various fractions of the DNP-prolyl type one, were observed, and are shown in Fig. 4. As clearly seen from the figure, pure DNP-alanine has its $\lambda_{1\,\mathrm{max}}$ at 353 mμ in *N* hydrochloric acid. As the content of DNP-proline in the former increases, the $\lambda_{1\,\mathrm{max}}$ is shifted by 2–3 mμ towards the longer wave length region per 0.1 mole addition. At the same time, the second absorption maximum at 265 mμ gradually lowers and finally disappears.

Using the *R* values (see p. 435) obtained from Fig. 4, it was intended to calculate the content of N-terminal proline in clupeine. The *R* values were shown to bear a linear relation to the content of the DNP-prolyl type peptide (Fig. 5). *R* values of several specimens of DNP-protamines and the N-terminal proline contents calculated therefrom are shown in Table II. It is evident from the table, that there are some variations in the values and the contents as the specimens differ with one another in date and place of capture of the fish material and in fraction of preparation.

On hydrolyses of several specimens of DNP-clupeine with hydrochloric acid, DNP-alanine and dinitrophenol were always found in the ether-soluble part of each hydrolysate (Tables III and VI). These were further confirmed by their mixed chromatography, and the spot of the DNP-alanine also by regenerating alanine through decomposition with ammonia. When several hydrolysates were carefully examined later on the two-dimensional paper chromatogram according to Levy, a trace of spot corresponding to DNP-proline was found in most cases together with the other two (see Table VI).

---

* Since in the earlier experiment by Hashimoto (*16*), the absorption was determined only in 1 per cent $NaHCO_3$ solution, the resulting curve of DNP-clupeine did not satisfactorily agree with that of DNP-amino acid used for control. Consequently the presence of proline coexisted in the N-terminal residues of clupeine was overlooked, and the conclusion was derived that all the N-termini corresponded only to amino groups. Moreover, if dinitrophenol was incompletely eliminated from the specimen, the influence of the substance on the absorption curve is not negligible in bicarbonate solution.

From the quantitative point of view, a considerable amount of DNP-alanine corresponding to 75–100 per cent of the content of the N-terminal amino group to be expected from the *R* value, and varying quantities of dinitrophenol were recovered through a Hyflo Super-Cel column chromatography (see Table VI). Since DNP-alanine is very resistant to acid hydrolysis* as shown in a control experiment in Table V, dinitrophenol always obtained in the above is considered to occur from DNP-prolyl peptides, as in the case of DNP-salmine. In a case of clupeine II which is the richest in the N-terminal proline, 0.41 mole of DNP-alanine and 0.33 mole of dinitrophenol were recovered from the hydrolysate (8 hours) through a Hyflo Super-Cel column. Under the same conditions, *ca.* 0.7 mole of dinitrophenol was obtained from one mole of DNP-salmine. When the former value (0.33 mole) of dinitrophenol is corrected with the latter (0.7 mole/mole of DNP-prolyl peptide), the present clupeine II should have 0.41 mole of of alanine and *ca.* 0.47 mole of proline in the N-terminal. These values are in considerable accord with those (0.6–0.5 mole of the terminal proline and 0.4–0.5 mole of such alanine) estimated from the *R* values (0.83–0.78) of the clupeine II (see Table II). As for clupeine I, 0.25 mole of the N-terminal proline was estimated from the *R* value (0.67), while 0.70 mole of the DNP-alanine (*i.e.*, 0.30 mole of DNP-proline) was actually recovered. Thus in clupeine, there can be seen a good agreement between the contents of alanine in the N-terminus estimated from the *R* value and from the actual recovery of DNP-alanine in the hydrolysate.

Close examination of the acid-soluble fraction of the hydrolysate of DNP-protamines with 6 *N* hydrochloric acid can not entirely exclude the the possibility, that an arginine residue sits in an extremely small quantity in their N-terminal. In view of the amount of absorption, due to the DNP-group, which remained in the aqueous layer after the hydrolysis, the amount of the substance (spot A or C in Fig. 7) apparently similar to DNP-arginine may be estimated to be only less than 0.05 mole** in our DNP-salmine*** and much lower in all our specimens of DNP-clupeine.

---

* In our recent experiment with DNP-alanylarginine, DNP-alanine has been recovered in an amount of *ca.* 90 per cent on hydrolysis (6 *N* HCl, 100°, 8 hours).

** Since the acid-soluble DNP-derivatives amounted to *ca.* 0.1 mole as shown in Table VI and they gave two spots (A and B in Fig. 7) of nearly equal amount by paper electrophoresis, the amount of substance similar to DNP-arginine is estimated to be 0.05 mole.

*** The *R* values of the DNP-proline (1.36 and 1.02 in 1 per cent $NaHCO_3$ and *N* HCl, respectively, as shown in Table II) are *ca.* 3 per cent lower than those of DNP-proline (1.40) and DNP-prolyl peptide (1.05) in 1 per cent $NaHCO_3$ given by Fraenkel-Conrat *et al.* (*19*). Those of the DNP-prolylarginine recently synthesized in our laboratory were 1.05 and 1.06 in 1 per cent $NaHCO_3$ and *N* HCl, respectively. Thus adopting 1.05 for the value of DNP-prolyl peptide, our specimen of DNP-salmine ($R=1.0_2$ in average) was calculated to contain *ca.* 5 per cent of α-amino group in the N-terminal. This content appears to be coincidentally similar to that of DNP-arginine estimated in the above.

When the ether-soluble fraction of acid hydrolysates of salmine and clupeine was studied by two-dimensional paper chromatography, an extremely faint spot was always found in the position corresponding to DNP-serine. If a usual correction factor (10 per cent) of DNP-serine (*8*, *31*) is applicable to this case*, the molar fraction of the serine in the N-terminus of the protamines should be less than 0.01 and may be neglected.

It is a usual way in N-terminal analysis of peptides containing proline in the terminus, to hydrolyze the DNP-peptides with concentrated hydrochloric acid and to identify DNP-proline, as proposed by Porter and Sanger (*8*). They reported that DNP-proline was recovered from DNP-salmine under the condition (105°, 16 hours in a sealed tube) (*8*). In our earlier case of the N-terminal analysis of protamines, the presence of proline in the terminus was carefully searched for, but in vain, by the hydrolysis of the DNP-protamines with concentrated hydrochloric acid (see Table III). Also in control experiments with DNP-proline, the decomposition was unexpectedly extensive, and DNP-proline could difficultly be recovered even in one hour hydrolysis. Two or three spots were always found instead on the paper chromatogram of the ether-soluble part of the hydrolysate of DNP-proline, one corresponding to an authentic specimen of dinitrophenol and the other two** having somewhat larger R_f values than that of DNP-proline and corresponding to none of DNP-amino acids to be derived from the protamines (see Tables III and I). The hydrolysis of DNP-salmine under the same condition gave also no DNP-proline but two of those spots, on the paper chromatogram too. From these results, the N-terminal residue of salmine was determined to be proline (*2*).

When the DNP-clupeine was similarly hydrolyzed, the spot corresponding to dinitrophenol in addition to that of DNP-alanine was found, but no spot with larger R_f value was seen. Consequently, it was then reported that all our specimens of clupeine had only alanine bnt no proline in the N-terminus* (*2*, *3*). It was also emphasized (*3*) that this result might be caused by the species difference between our specimens and those of the European herring which were said to have only proline in the N-terminal residue. This conclusion needs to be somewht corrected, as the proline besides alanine has now been shown to be present in the N-terminus. It has been further confirmed by the isolation of several peptides such as DNP-prolylarginine, DNP-alanylarginine, DNP-prolylarginylarginine and DNP-alanylarginylarginine from the tryptic hydrolysate of DNP-clupeine, that a considerable amount of fraction of clupeine has proline in the N-terminal**. From the amount of the peptides isolated above, clupeine I is calculated to contain *ca.* 0.13 mole of proline as the N-terminal residue and clupeine II. *ca.* 0.50 mole. The latter value agrees with those (0.6–0.5 mole) obtained by two methods described in this paper, while the former is but a half of those (0.25 and 0.30 mole). The reason for this discrepancy is not clear.

---

* The results of Levy *et al.* (*32*) and Bell *et al.* (*33*) have shown that in some cases DNP-seryl peptides suffer extensive destruction under usual acid hydrolysis. Bell (*33*) and Woolley *et al.* (*34*) have used a mixture of conc. HCl and glacial acetic acid (1 : 1, *v/v*) as a hydrolyzing agent in their structure studies of DNP-seryl peptides, for the purpose of increasing the recovery of DNP-serine. Though we have also compared both agents, 6 *N* HCl and the above mixture, for the hydrolysis of the DNP-derivatives of tryptic digest of clupeine, the recovery of DNP-serine was almost the same with each other. Thus, in our case, no greater value than 10 per cent would be necessary to consider as the correction factor of DNP-serine.

** Recently Scanes and Tozer (*35*) have studied the hydrolysis of DNP-proline with concentrated HCl and found, that the decomposition of the DNP-proline was very large and a considerable amount (*ca.* 0.5 mole in total per mole of DNP-proline) of two kinds of DNP-derivatives containing chlorine, called "hydrolytic product A" and "B", were produced. They have also shown that, on further hydrolysis of these products, proline and dinitrophenol were regenerated quantitatively. The spots with larger R_f values found in our earlier studies correspond probably to their hydrolytic products. Our studies following and modifying their experiment using conc. HCl (2 and 4 hours) and conc. HCl-AcOH (1 : 1, *v/v*, 4 and 8 hours) also showed similar results, yielding only 0.05–0.02 mole of DNP-proline but 0.50–0.30 mole in total of the hydrolytic products A and B.

The clupeine prepared from the European herring has hitherto been reported to have only proline in the N-terminal residue, as shown by Felix (*11*) and Waldschmidt-Leitz (*36*) with specimens from *Clupea harengus.* Recently Tozer and Scanes (*37*), however, have found that a specimen of clupeine from undescribed origin has alanine besides proline in the N-terminus***. The latter has also shown that a fraction containing only alanine in the N-terminal was obtained and had a more simplified amino acid composition. They have pointed out that the ratio of the N-terminal proline to the alanine possibly varies with the fractionation method of clupeine. In our case of clupeine fractionated by making use of the solubility difference of its picrate in aqueous acetone, the prolyl peptides seem to be rather concentrated in the less soluble fraction (compare clupeines II, II-1 and II-2 in Table II). However in this respect, much more experiments are needed to obtain a settled conclusion. Recent investigation of fractionation in our laboratory with alumina chromatography and counter-current distribution technique has begun to show some interesting results (*39*).

Our result that proline in addition to alanine was found in the N-terminal residue of our specimens of clupeine, brings forward the following problem which remains to be clarified in future. Is the result properly characteristic for clupeine or not? If not, it may be within the range of possibility, that the fish material we used is a mixture of different species or a hybrid, or the testes are contaminated with immature ones, or else the decomposition of clupeine is partly resulted by the effect of some proteases in the tissue, during the course of preparation after the capture of fish.

---

* It may be considered to be reasons for overlooking the proline in the N-terminus in our earlier experiment, that the specimens then used (clup. I and clup. III) contained smaller amount of proline (0.25 and 0.36 mole), an incomplete elimination of dinitrophenol from the DNP-protamine gave some influences upon the result, and the method by Porter and Sanger was not generally applicable to the identification of DNP-prolyl peptides.

** Confer Ando *et al.* (*4*). Details are in preparation.

*** Similar results have recently been found with clupeine from *Clupea harengus* by K. Felix (*38*).

To answer that question, it is necessary to reinvestigate problems carefully using new material to be obtained from each sperm or single testis of complete maturity.

SUMMARY

1. N-terminal residues of salmine obtained from Japanese salmon (*Oncorhynchus keta*) and clupeine from Japanese herring (*Clupea pallasii*) were studied by the DNP-method. DNP-Protamines have been prepared by three methods.

2. The absorption spectra of these DNP-protamines were studied over the wave-length region of 450–230 mμ. Based on the curves, it was inferred that the N-terminal group of salmine is exclusively occupied by an imino group (Fig. 2), while that of clupeine by both amino and imino groups (Fig. 3).

3. *R* values (the ratio of optical densities at 390/360 mμ of absorption curves) of some DNP-derivatives were calculated from their absorption curves, and a mixed ratio of the N-terminal amino and imino groups of clupeine was estimated (Table II and Fig. 5). Of five specimens of clupeine used, the content of the N-terminal imino group amounted to 0.60–0.23 mole per mole of the N-terminal group. Thus, some differences were found among specimens, as either the date or place of capture of fish or the fraction of clupeine differs.

4. The DNP-N-terminal residues of protamines were identified and determined by chromatographic methods after acid hydrolysis (Table III and VI). A considerable amount of dinitrophenol together with a small quantity of DNP-proline was recovered from the hydrolysate of DNP-salmine, and the N-terminal residue was thus confirmed to be occupied by proline. From DNP-clupeine, DNP-alanine corresponding to 75–100 per cent of the amount of the N-terminal amino group estimated from the *R* value, and various amounts of dinitrophenol were recovered, and it was concluded that the N-terminal residue was occupied by both alanine and proline to various extents as the specimens differ.

5. The possibility that a few amino acids other than those demonstrated above may occupy an extremely slight fraction of the N-terminal residue of the protamines could not completely be eliminated.

6. Some considerations were made concerning the meaning of the result that both alanine and proline were found in the N-terminus of clupeine.

The authors are indebted to Drs. K. Imahori and K. Iwai for their interest and valuable discussion, and to Prof. Y. Hirata of Nagoya University for his kindness of making us the necessary literature available in our earlier experiment of this work. The present work was aided in part by the Scientific Research Grant from the Ministry of Education, to whom our thanks are also due.

REFERENCES

(*1*) Ando, T., Iwai, K., and Kimura, M., *J. Biochem.*, **45**, 27 (1958)
(*2*) Ando, T., Ishii, S., Hashimoto, C., Yamasaki, M., and Iwai, K., *Bull Chem. Soc. Japan*, **25**, 132 (1952)
(*3*) Ando, T., Iwai, K., Yamasaki, M., Hashimoto, C., Kimura, M., Ishii, S., and Tamura, T., *Bull. Chem. Soc. Japan*, **26**, 406 (1953)
(*4*) Ando, T., Abukumagawa, E., Nagai, Y.. and Yamasaki, M., *J. Biochem.*, **44**, 191 (1957)
(*5*) Ando, T., Nagai, Y., and Fujioka, H., *J. Biochem..* **44**, 779 (1957)
(*6*) Ando, T., Ishii, S., Yamasaki, M., Iwai, K., Hashimoto, C., and Sawada, F., *J. Biochem.*, **44**, 275 (1957)
(*7*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)
(*8*) Porter, R. R., and Sanger, F., *Biochem. J.*, **42**, 287 (1948)
(*9*) Perrone, J. C., *Nature*, **167**, 513 (1951)
(*10*) Sanger, F., *Biochem. J.*, **45**, 563 (1949)
(*11*) Felix, K., and Krekels, A., *Z. physiol. Chem.*, **295**, 107 (1953)
(*12*) Levy, A. L., *Nature*, **174**, 126 (1956)
(*13*) Blackburn, S., and Lowther, A. G., *Biochem. J.*, **48**, 126 (1951)
(*14*) Acher, R., and Crocker, C., *Biochim. Biophys. Acta*, **9**, 704 (1952)
(*15*) Biserte, G., and Osteux, R., *Bull. soc. chim. biol.*, **33**, 50 (1951)
(*16*) Hashimoto, C., *Bull. Chem. Soc. Japan*, **28**, 385 (1955)
(*17*) Redfield, R. R., and Barron, E. S. G., *Arch. Biochem. Biophys.*, **35**, 443 (1952)
(*18*) Chinard, F. P., *J. Biol. Chem.*, **199**, 91 (1952)
(*19*) Confer Niu, C.-I, and Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, **77**, 5882 (1955)
(*20*) Phillips, D. M. P., *Biochem. J.*, **60**, 403 (1955)
(*21*) Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., "Recent Developments in Techniques for Terminal and Sequence Studies in Peptides and Proteins", in "Methods of Biochemical Analysis" (edited by Glick, D.), Interscience Publishers, Inc., New York, Vol. II, p. 374 (1955)
(*22*) Lowther, A. G., *Nature*, **167**, 767 (1951)
(*23*) Mills, G. L., *Biochem. J.*, **50**, 707 (1952)
(*24*) Schroeder, W. A., and LeGette, J., *J. Am. Chem. Soc.*, **75**, 4612 (1953)
(*25*) Velick, S. F., and Udenfriend, S., *J. Biol. Chem.*, **191**, 233 (1951)
(*26*) Monier, R., Jutisz, M., *Biochim. Biophys. Acta*, **14**, 551 (1954)
(*27*) Mason, H. S., Peterson, E. W., *J. Biol. Chem.*, **212**, 485 (1955)
(*28*) Schramm, G., and Braunitzer, G., *Z. Naturforschg.*, **8b**, 61 (1953)
(*29*) Fraenkel-Conrat, H., and Singer, B., *J. Am. Chem. Soc.*, **76**, 180 (1954)
(*30*) Schroeder, W. A., Kay, L. M., LeGette, J., Honnen, L., and Green, F. C., *J. Am. Chem. Soc.*, **76**, 3556 (1954)
(*31*) Woodin, A. M., *Biochem. J.*, **63**, 576 (1956)
(*32*) Levy, A. L., and Li, C. H., *J. Biol. Chem.*, **213**, 487 (1955)
(*33*) Shepherd, R. G., Howard, K. S., Bell, P. H., Cacciola, A. R., Child, R. G., Davies, M. C., English, J. P., Finn, B. M., Meisenhelder, J. H., Moyer, A. W., and van der Scheer, *J. Am. Chem. Soc.*, **78**, 5051 (1956)
(*34*) Merrifield, R. B., and Woolley, D. W., *J. Am. Chem. Soc.*, **78**, 358 (1956)

(35) Scanes, F. B., and Tozer, B. T., *Biochem. J.*, **63**, 282 (1956)
(36) Waldschmidt-Leitz, E., Kühn, K., Zinnert, F., *Experientia*, **7**, 183 (1951)
(37) Scanes, F. S., and Tozer, B. T., *Biochem. J.*, **63**, 565 (1956)
(38) Felix, K., Krekels, A., and Rick, W., *Trans. Faraday Soc.*, **53**, 252 (1957)
(39) Sawada, F., and Ando, T., The Joint Meeting of Chemical Societies in Japan, Tokyo, Nov. 23, 1957. See also Ando, T., Iwai, K., Ishii, S., Yamasaki, M., Tobita, T., Sawada, F., Nagai, Y., Fujioka, H., Kimura, M., Sato, M., Abukumagawa, E., and Kawanishi, Y., Symposium on Chemical Structure of Proteins, Tokyo, Oct. 28, 1957.

The Journal of Biochemistry, Vol. 45, No. 6, 1958

STUDIES ON PROTAMINES

IV. THE BASIC PROTEINS OF THE TESTIS CELL NUCLEI OF ADULT AND IMMATURE RAINBOW-TROUTS IN BREEDING SEASON*

BY TOSHIO ANDO AND CHIEKO HASHIMOTO

(*From the Institute of Science and Technology,*
University of Tokyo, Tokyo)

(Received for publication, December 28, 1957)

Sperm nuclei obtained from sperm and testes of some kinds of mature fish consist mostly of nucleoprotamine in which protamine combines as salt with nucleic acid. Studies on the chemical structure of protamines isolated from testis nuclei of herring (*Clupea pallasii*) and salmon (*Oncorhynchus keta*), both in maturity, have partly been reported in a series of this work (*1–3*).

In order to see whether there is any difference in nature of the basic proteins in sperm or testis nuclei between an adult and an immature fish, sperm and testes of rainbow-trouts (*Salmo irideus*) were used in this study. It is reasons why the fish of this species was selected for the present investigation, that fresh material can be easily collected in every time of seasons, and it can be of a pure line and of an known age.

Thus in this work, spermatozoa obtained from sperm and testes of rainbow-trouts of 5 years old (adult) and testes of those of 2 years old (immature), both in breeding season (January-March), were first compared by observing with photo- and electronmicroscopes. Sperm nuclei were then isolated from the spermatozoa and their elementary composition was studied comparatively. Lastly, the basic proteins were separated from the isolated nuclei and their chemical structures were investigated comparing with each other.

EXPERIMENTAL

a) *Material*—Sperm and testes of rainbow-trouts (*Salmo irideus*) during a breeding season were obtained from the fish hatchery of Tokyo city at Okutama, Tokyo.** The testes were excised from 2 and 5 year old trouts, and the sperm was obtained by stripping 5 year old trouts. Within three hours after collection, the material was transported to our laboratory in a container packed with natural ice or dry ice. All the treatments described below for the isolation of nuclei and the preparation of basic proteins were made at the temperature of 0 to 4°.

---

* Partly presented at the 7th and 8th Annual Meetings of the Chemical Society, Japan (Tokyo, April 1954 and 1955) and at the International Symposium on Enzyme Chemistry (Tokyo, October, 1957).

** We are much indebted to Mr. S. Ohuchi, the superintendent of Okutama branch of the Tokyo City Fisheries Experimental Station for the material.

b) *Isolation of Sperm Nuclei*—The methods for the isolation of sperm nuclei by Mirsky and Fischer (*4*) were investigated, and a partly modified method was employed for the present study. The sperm or testes were homogenized and stirred vigorously in a large volume of Ringer's solution for fresh-water fish (NaCl : 0.65 per cent, KCl : 0.014 per cent, $CaCl_2$: 0.012 per cent, $NaHCO_3$: 0.012 per cent, NaH_2PO_4 : *ca.* 0.001 per cent) or of 0.14 *M* NaCl. After the suspension was filtered through two sheets of cotton cloth, it was centrifuged. The precipitate thus obtained was again washed with some Ringer's solution and then twice with Ringer's solution containing 0.2 per cent sodium citrate. It was repeatedly washed with Ringer's solution in which alcohol was contained in successively higher concentrations. Finally, it was washed with absolute alcohol and dried with ether or acetone.

These treatments were made with the utmost care, for the cell nuclei in the immature testes were very unstable. Following the above process of isolation of sperm nuclei with photo- and electronmicroscopic observations, it was seen that the sperm and testis cell nuclei always remained without any apparent deformation after the first treatment of centrifugation (*5*).

c) *Preparation of Basic Proteins from the Isolated Nuclei*—The sperm nuclei, obtained after washing with Ringer's solution containing 0.2 per cent sodium citrate, was vigorously homogenized in 2 *M* NaCl. To the saline solution in which nucleoprotamine was extracted, a saturated solution of cupric sulfate was added to precipitate nucleic acid as cupric salt. By adding *M*/8 - sodium picrate to the supernatant after centrifugation, the basic protein was precipitated as its picrate. On the other hand, another method was also adopted, in which the basic protein was extracted with 0.2 *N* HCl from the isolated nuclei and was precipitated as picrate. After centrifugation and drying, the basic protein picrate was dissolved in 67 per cent aqueous acetone solution. By dropwise addition of 2 *N* sulfuric acid, it was then converted to sulfate, which was further purified by repeated precipitation with water and alcohol to yield colorless glittering powder.

d) *Composition Analyses of the Isolated Nuclei and the Basic Proteins*—The amino acid composition of each specimen of the isolated nuclei and purified basic protein sulfates was investigated qualitatively and quantitatively. For the identification of the component amino acids, 10 mg. each of specimens was hydrolyzed with 20 per cent hydrochloric acid at 110° in sealed tube for 32 hours, and the product was subjected to two-dimensional paper chromatography using phenol- ammonia and *n*-butanol-formic acid systems as solvents. For the quantitative determination of the component basic amino acids, the method of Ishii (*5*) in our laboratory was used, while other amino acids were determined by the method of Moore and Stein (*7*). The phosphorus content was determined by the methods of King (*8*) and Schmidt-Thannhauser (*9*), and the arginine content by the method of Sakaguchi (*10*).

e) *Structure Analysis of the Basic Proteins*—2,4-Dinitrophenyl(DNP) derivative of each basic protein sulfate was prepared according to the method of Sanger (*11*). The DNP-protein was hydrolyzed by heating in sealed tube at 105° for 16 hours with 20 per cent hydrochloric acid and 10 hours with 35 per cent hydrochloric acid, respectively. DNP-amino acids yielded were identified and determined by means of two-dimensional paper chromatography according to Levy (*12*).

On the other hand, each of the DNP-basic protein sulfates was purified by the method described in a previous paper (*13*), using an activated alumina column. Besides, the purification using columns of Amberlite XE-64 (H-form, 0.9 × 15 cm.) and IR-4B (0.9 × 10 cm.) was also attempted. By determining the absorption spectrum of the purified

DNP-basic protein sulfate, its N-terminal amino acid was studied and its molecular weight was also estimated. Furthermore, phenylthiocarbamyl(PTC) basic protein sulfate was prepared and hydrolyzed with anhydrous formic acid to yield the phenylthiohydantoin (*14*). The latter was identified by using one-dimensional paper chromatography by Landman and Sjöquist (*15*).

As regards the identification of the C-terminal residue of the basic protein, the hydrazinolysis method of Akabori (*16*) was adopted. On the other hand, carboxypeptidase recrystallized seven times and an enzyme fraction, which was obtained by Dr. Tobita in our laboratory in the course of the preparation of the former enzyme and shown to contain basic carboxypeptidase (*17*) besides, using carbobenzoxy-glycyl-L-arginine, were also used. Of course, they were treated with DFP (diisopropyl fluorophosphate) just prior to the use. The enzyme fraction containing basic carboxypeptidase was incubated with the basic protein for 2, 8, and 24 hours in 0.02 *M* Veronal buffer containing 0.1 *N* sodium chloride of pH 7.5 at 25°. Amino acid released was identified on one-dimensional paper chromatogram (*n*-butanol- formic acid system) and also according to Levy (*12*) after dinitrophenylation.

RESULTS AND DISCUSSION

Typical figures in a number of 5 (adult) and 2 (immature) year old

TABLE I

Pictures of 5 and 2 Year Old Rainbow-Trouts in Breeding Season

	Body length	Body width	Body weight	Weight of one testis
	(*cm.*)	(*cm.*)	(*g.*)	(*g.*)
5 year old	43	11	830	6.5 11.3
2 year old	27	7.5	300	2.5 5.5

Each of these fish is of a typical type respectively among rainbow-trouts of 5 and 2 years old used for this work, which are close to an average of 50 fish.

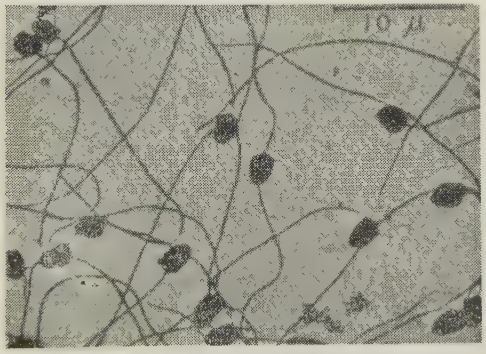


FIG. 1. An electronmicrograph of spermatozoa obtained from a rainbow-trout of 5 years old during breeding season.

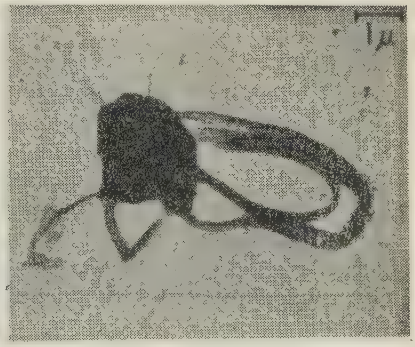


FIG. 2. An electronmicrograph of a spermatozoon obtained from a rainbow-trout of 2 years old during breeding season.

rainbow-trouts in breeding season are shown in Table I. The conspicuous morphological features of spermatozoa obtained from their sperm and/or testes were observed by means of an electronmicroscope*. As seen from Figs. 1 and 2, no difference was found among spermatozoa obtained from trouts of 5 and 2 years old. Details of microscopic and chemical analytical studies on spermatozoa will be later reported elsewhere by one of the authors.

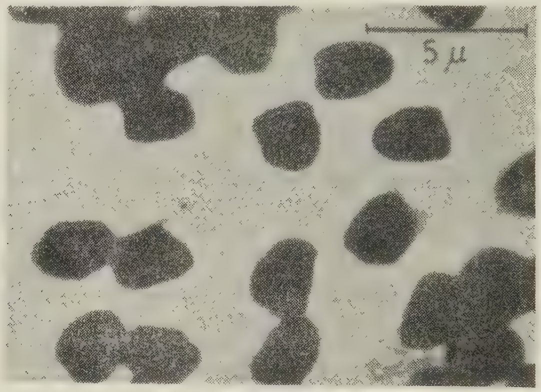


FIG. 3. An electronmicrograph of sperm nuclei isolated from one testis of a rainbow-trout of 5 years old during breeding season.

TABLE II

*Composition of Isolated Sperm Nuclei***

	Yield of isolated sperm nuclei***	Total N†	Total P††	N/P	PNA-P††	Arg†††	P/Arg	Yield of basic protein
	(%)	(%)	(%)		(%)	(%)		(%)
5 year old rainbow-trout	6.6	20.6	5.6_3	3.6	0.0	$33._1$	$1:1.0_4$	42
2 year old rainbow-trout	6.0	20.6	5.3_5	3.9	0.0	$30._5$	$1:1.0_1$	47

** Values are given in percentage of the weight of isolated and dried nuclei.

*** Values are given in percentage of the weight of isolated and dried nuclei per one fresh testis.

† Obtained by Kjeldahl's method.

†† Obtained by King's method (*8*) and Schmidt and Thannhauser's method (*9*).

††† Obtained by Sakaguchi's method (*10*).

---

* We are indebted to Mr. S. Sakata in the laboratory of Electron Microscopy, Tokyo University, for the electronmicroscopic techniques.

The process of isolating nuclei from sperm or testis was also followed by observation with a photomicroscope, and it was found that only sperm nuclei were isolated without any deformation. Fig. 3 shows an electronmicrograph of the isolated sperm nuclei. The results of analyses of these sperm nuclei are given in Table II. As to the amino acid composition of these isolated sperm nuclei, lysine, glutamic and aspartic acids, and threonine were found besides the amino acids of the basic proteins shown in Table III. Almost no difference could be found in the composition of sperm nuclei isolated either from sperm (stripped from 40–50 fish) and testes (excised from 1–3 fish) of 5 year old trouts, or from testes of 2 year old trouts (excised from 2–3 fish), all in their breeding season (January-March). As no PNA was present at all in any sperm nucleus, it was seen that the sperm nuclei almost consisted of DNA and basic protein. The content of DNA may be estimated from the phosphorus content. Furthermore, it is of interest, that the molar ratio of phosphorus in DNA to arginine in basic protein gave almost one (*18*).

TABLE III

Amino Acid Composition of Basic Proteins Obtained from Sperm Nuclei of 5 and 2 Year Old Rainbow-Trouts*

	Total N**(%)	Arg	Ser	Gly	Val	Ala	Ileu	Pro	(NH$_3$)	Recovery
5 year old	23.0	89.$_6$	3.3	1.9	1.7	0.6	0.3	2.8	(0.5)	100.$_2$
2 year old	22.1	91.$_1$	3.1	2.1	2.0	0.7	0.3	2.3	(1.1$_4$)	101.$_6$

* Determined by an ion-exchange chromatographic method (*6*, *7*), according to the advices of Drs. Ishii and Sato. Values for amino acids are shown in amino acid N as percentage of total N.

** Obtained by Kjeldahl's method, for basic protein sulfates.

The amino acid composition and the absorption curve of each basic protein sulfate prepared from the isolated sperm nuclei are shown in Table III and Fig. 4 (curve A), respectively. These results show that no remarkable difference can be found in the amino acid composition and in the absorption spectra of both basic proteins obtained from 5 and 2 year old fish. The absorption curve of the present protein was found to be the same as that of clupeine and salmine previously reported (*3*). The amino acid composition was similar to that of iridine from a rainbow-trout (*salmo irideus*) obtained by Felix *et al.* (*18*), except lysine. The latter amino acid, lysine, has been reported to be present in the protamine obtained by German investigators, while, in contrast, it was absent in both of our specimens of basic proteins obtained from 5 and 2 year old rainbow-trouts during breeding season.

Results of some structure analyses of the basic proteins are summarized in Table IV. On hydrolysis with 20 per cent hydrochloric acid, both DNP-basic proteins gave dinitrophenol and dinitroaniline in the hydrolysates, while those, DNP-proline and other DNP-derivative*** were found by the hydrolysis with 35 per cent hydrochloric acid. These DNP-derivatives were the same as those produced by hydrolyzing DNP-proline for control under

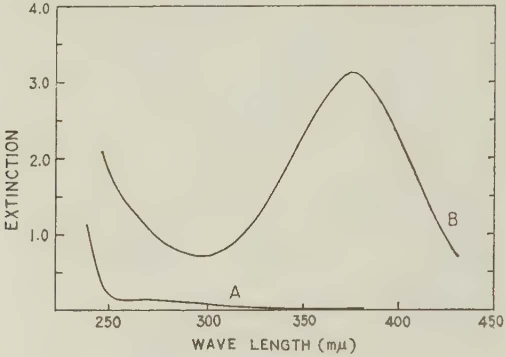


FIG. 4. Absorption spectra of basic protein sulfate (curve A) and its DNP-derivative (curve B). Curve A: 0.5 per cent aqueous solution of each basic protein obtained from 5 or 2 year old trout. Curve B: a solution containing 1 g. of each DNP-basic protein in 1 liter of 1 per cent aqueous $NaHCO_3$.

TABLE IV

Structure Analysis of the Basic Protein

	C-Terminal residue			N-Terminal residue	Molecular weight
	CPase* digestion	Basic CPase** digestion	Hydrazinolysis	DNP method and PTC method	Estimated from absorption spectra of DNP-protein
5 year old rainbow-trout	resistant	Arg	di-DNP-Orn	Pro	5,800† 5,000††
2 year old rainbow-trout	resistant	Arg	di-DNP-Orn	Pro	5,200††

* Carboxypeptidase

** A fraction containing so-called basic carboxypeptidase was used.

† For the absorption measurement, a specimen of DNP-protein sulfate was used after purification through a column of activated alumina (molecular weight as free base).

†† A specimen was used after purification through a column of Amberlite XE-64.

*** This had an absorption spectrum corresponding to that of DNP-monoamino acid and was also obtained in the hydrolysate of DNP-proline with 35 per cent HCl (*19*).

the same condition. On the other hand, the ultraviolet absorption spectra of purified DNP-basic proteins were observed and the results are shown in Fig. 4 (curve B). The absorption curve, having only one maximum at 375 mμ, is of the same type as that of salmine. Thus the ratio of optical density at 390 mμ to that at 360 mμ was found to be 0.99–1.0 (*2*). In addition to this, only the phenylthiohydantoin of proline was identified in the hydrolysates of both PTC-basic proteins by paper chromatography. All these results may lead to the conclusion that the N-terminal residue of these basic proteins is exclusively occupied by proline.

As for the C-terminal residue of each of these basic proteins, there have been obtained some results which support arginine to be present in the greater part of the termini. By the hydrazinolysis method of Akabori *et al.* (*16*), a considerable part at least of the C-terminal residues of these proteins was found to be occupied by arginine, as indicated in the yield of di-DNP-ornithine.* On the other hand, when these basic proteins were incubated with DFP-treated carboxypeptidase, they were entirely resistant to the enzyme.* Such a result has also been obtained in our laboratory with other protamines, clupeine and salmine, which are both proved to have arginine in their C-termini. Furthermore, when the basic protein obtained from 5 year old trout was incubated for 2 and 8 hours with a DFP-treated enzyme fraction prepared and shown by Dr. Tobita in our laboratory to contain basic carboxypeptidase (*17*), it was found that only arginine was surely released from the protein in any period of incubation.

The molecular weight of each basic protein may be calculated, assuming that the molar absorption coefficient of DNP-group in the DNP-basic protein at its absorption maximum (375 mμ) is equal to that of DNP-proline at its maximum (387 mμ). Thus the molecular weights of basic proteins obtained from 5 and 2 year old trouts were estimated both to be about 5,000.

All the results described in the above show, that no difference could be found so far between the nature of each basic protein obtained from sperm and/or testis nuclei of adult (5 year old) or immature (2 year old) rainbow-trouts during breeding season. Both of these basic proteins can thus be regarded with the highest probability as the same protamine, iridine.

SUMMARY

The sperm nuclei were isolated from sperm and testes of adult rainbow-trouts (5 years old) and testes of immature ones (2 years old) during breeding season. Basic protein was prepared from both kinds of the isolated sperm and testis nuclei, respectively. From results of the composition analyses of the nuclei (nitrogen, phosphorus, arginine and amino acids) and the basic proteins (nitrogen and amino acids), no difference could be found between

---

* The hydrazinolysis and the carboxypeptidase digestion experiments were kindly made by Drs. Y. Kawanishi and T. Tobita, respectively, in our laboratory. Details on the C-terminal residues of protamines will be fully reported elsewhere from our laboratory.

5 and 2 year old trouts.

Based upon some structure analyses, both the basic proteins obtained from these trouts were concluded to be consisted of the same protamine, iridine, having molecular weight of ca. 5,000, in which the N-terminal residue was found to be exclusively proline, while the C-terminal to be for the most part arginine.

The authors are indebted to Drs. S. Ishii and M. Yamasaki of this laboratory for their valuable suggestion and to Dr. M. Sato for help in amino acid determination.

This work was supported in part by the Scientific Research Grant from the Ministry of Education.

REFERENCES

(*1*) Ando, T., Ishii, S., Hashimoto, C., Yamasaki, M., and Iwai, K., *Bull. Chem. Soc. Japan*, **25**, 132 (1952); Ando, T., Iwai, K., Yamasaki, M., Hashimoto, C., Kimura, M., Ishii, S., and Tamura, T., *Bull. Chem. Soc. Japan*, **26**, 406 (1953); Ando, T., Nagai, Y., and Fujioka, H., *J. Biochem.*, **44**, 779 (1957)

(*2*) Ando, T., Abukumagawa, E., Nagai, Y., and Yamasaki, M., *J. Biochem.*, **44**, 191 (1957)

(*3*) Ando, T., Ishii, S., Yamasaki, M., Iwai, K., Hashimoto, C., and Sawada, F., *J. Biochem.*, **44**, 275 (1957)

(*4*) Pollister, A. W., and Mirsky, A. E., *J. Gen. Physiol.*, **30**, 101 (1946); Daly, M. M., Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **34**, 439, (1951); Fischer, H., Kreuzer, L., *Z. physiol. Chem.*, **293**, 176 (1953)

(*5*) Hashimoto, C., and Sakata, S., The 11th Ann. Meeting of the Society of Electron-Microscopy, Japan (Yokohama, May, 1955)

(*6*) Ishii, S., *J. Biochem.*, **43**, 531 (1956)

(*7*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951)

(*8*) King, E. J., *Biochem. J.*, **26**, 292 (1932)

(*9*) Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945)

(*10*) Sakaguchi, S., *J. Biochem.*, **37**, 231 (1950); **38**, 91 (1951)

(*11*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)

(*12*) Levy, A. L., *Nature*, **174**, 126 (1954)

(*13*) Hashimoto, C., *Bull. Chem. Soc. Japan*, **28**, 385 (1955)

(*14*) Edman, P., *Acta Chem. Scand.*, **4**, 283, (1950); Thompson, E. O. P., *J. Biol. Chem.*, **208**, 565 (1954)

(*15*) Landmann, W. A., Drake, M. P., and Dillaha,. J., *J. Am. Chem. Soc.*, **75**, 3638 (1953); Sjöquist, J., *Acta Chem. Scand.*, **7**, 447 (1953)

(*16*) Akabori, S., Ohno, K., Ikenaka, T., Nagata, A., and Haruna, I., *Proc. Jap. Acad.*, **29**, 561 (1953); Ohno, K., *J. Biochem.*, **40**, 621 (1953)

(*17*) Folk, J. E., *J. Am. Chem. Soc.*, **78**, 3541 (1956)

(*18*) Felix, K., Fischer, H., Krekels, A., and Mohr, R., *Z. physiol. Chem.*, **287**, 223 (1951)

(*19*) Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., "*Methods of Biochemical Analysis*", Vol. **II**, 359 (1955); Scanes, F. S., and Tozer, B. T., *Biochem. J.* **63**, 282 (1956)

The Journal of Biochemistry, Vol. 45, No. 6, 1958

STUDIES ON THE BINDING OF LABELED L-THYROXINE WITH BOVINE PLASMA ALBUMIN SUBFRACTIONS

Studies on the heterogeneity of serum albumin have been recently carried out by various methods such as column chromatography (*1–3*), difference in solubility (differential salting-out (*4–6*), fractional crystallization (*7–12*), turbidometric analysis (*13*, *14*)), diffusion constant (*15*, *16*), optical rotation of albumin solution (*17*, *18*), and electrophoresis (*19–29*), *etc.* However, only a few investigations (*7*, *20*, *21*) have been made on biochemical properties of albumin subfractions. In view of the fact that thyroxine is likely to bind with serum albumin (Gordon (*30*), Larson (*31*, *32*, *35*), Robbins (*33*), Slater (*34*) and others), the author studies on the binding of I^{131}-labeled L-thyroxine with bovine plasma albumin subfractions (Armour's bovine plasma albumin) by using Tiselius' hydroxylapatite column (*1*).

The I^{131}-labeled L-thyroxine was prepared according to Gleason's method (*36*). The purity of labeled L-thyroxine was confirmed by paper-chromatography (solvent: *tert-iso*-amyl alcohol saturated with 2 *N* NH_4OH solution (*36*)). 30 mg. of the bovine plasma albumin was dissolved in 10.0 ml. of 0.02 *M* sodium phosphate buffer solution pH 6.80 in which 160 μg. of labeled sodium salt of L-thyroxine was suspended, and incubated at 35.1° for 40 minutes. The incubated protein solution was centrifuged at 3,500 r.p.m. for 30 minutes in order to remove insoluble labeled L-thyroxine and the supernatant solution was dialyzed for about 24 hours at 2° against 500 ml. of 0.02 *M* sodium phasphosphate buffer pH 6.8. The dialyzed solution was chromatographed at 8–12° by using hydroxylapatite column (15 mm × 13 cm.) as an adsorbent and 0.02 *M*, 0.04 *M*, 0.07 *M*, 0.11 *M* and 0.4 *M* sodium phosphosphate buffer (pH 6.8) as an eluent. The elution rate was about 25–30 ml. per hour and the effluent was fractionated into each 3.5 ml. fraction by using fraction collector. The protein content was analyzed by the extinction at 280 mμ in 1 cm. cell in a Beckman spectrophotometer. The labeled L-thyroxine was measured by Geiger-Müller counter after drying 2.0 ml. of each fraction at 60° to obtain uniform film.

An example of the results is illustrated in Fig. 1, in which open and filled circles show protein content and radioactivity (counts per minute), respectively. Fig. 1 shows that the binding ability of 2nd subfraction with L-thyroxine was stronger than that of other subfractions, and that a peak of protein content of 1st subfraction was not coincident with that of L-thyroxine.

When lager amount of L-thyroxine was added to 30 mg. of bovine plasma albumin which was dissolved in 0.01 *M* sodium phosphate buffer pH 6.8, it was found that amounts of bound L-thyroxine in each subfraction were almost proportional to protein content, and moreover a peak of protein of 1st subfraction was coincident with that of L-thyroxine. When one part of this 1st subfraction was incubated with a solution of 25 mg. of bovine plasma albumin (0.01 *M* sodium phosphate buffer, pH 6.8), it was also observed that the result obtained was very similar to that of Fig. 1.

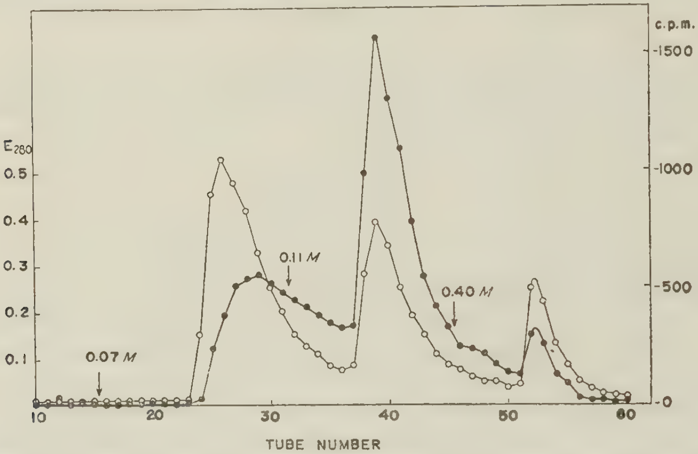


FIG. 1. Open circles show the chromatogram of bovine plasma albumin. The abscissa and the ordinate (left) show tube number of fraction collector (one tube 3.5 ml.) and extinction at 280 mμ, respectively. Elution was carried out at 8–12° by using the following buffer; 0.02 *M*, 0.04 *M*, 0.07 *M*, 0.11 *M* and 0.4 *M* sodium phosphate buffer, pH 6.8. Filled circles show the radioactivity of I^{131}-labeled L-thyroxine (counts per minute). The ordinate (right) shows the radioactivity of L-thyroxine.

From these experimental facts, it may be concluded that the binding ability of 2nd albumin subfraction is stronger than that of other subfractions. However, the detailed mechanism of this phenomenon will not be clear until more information is available on the molecular structure of these protein subfractions.

The author wishes to thank Prof. A. Inouye (Department of Physiology, Kobe Medical College) and the research group for thyroid gland (Prof. M. Miyake and Prof. G. Wakisaka, Department of Internal Medicine, Faculty of Medicine, Kyoto University) for their helpful advices. The author also expresses gratitude to the research group of thyroid gland, Prof. C. Nagaishi and Prof. S. Tsuji (Institute for Tuberculosis, Faculty of Medicine, Kyoto University) for their permission to use the G. M. counter and Beckman spectrophotometer.

REFERENCES

(*1*) Tiselius, A., Hjerten, S., and Levin, Ö., *Arch. Biochem.* and *Biophys.*, **64**, 217 (1956)
(*2*) Sober, H. A., Gutter, F. J., Wykoff, M. M., and Peterson, E. A., *J. Am. Chem. Soc.*, **78**, 756 (1956)
Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1956)

(*3*) Boman, H. G., and Westland L. E., *Arch. Biochem. Biophys.*, **64**, 217 (1956)
(*4*) Derrien, Y., *Biochim. Biophys. Acta*, **8**, 631 (1952)
(*5*) Derrien, Y., Steyn-Parvé, E. P., Cotte, M., and Laurent, G., *Biochim. Biophys. Acta*, **9**, 49 (1952)
(*6*) Steyn-Parvé, E. P., and Van Den Hout, A. J., *Biochim. Biophys. Acta*, **10**, 320 (1953)
(*7*) Kendall, F. E., *J. Biol. Chem.*, **138**, 97 (1941)
(*8*) McMeekin, T. L., *J. Am. Chem. Soc.*, **61**, 2884 (1939)
(*9*) McMeekin, T. L., *J. Am. Chem. Soc.*, **62**, 3393 (1940)
(*10*) Hewitt, L. F., *Biochem. J.*, **30**, 2229 (1938)
(*11*) Kekwick, R. A., *Biochem. J.*, **32**, 552 (1938)
(*12*) Cohn, E. J., Hughes, W. L. Jr., and Weare, T. H., *J. Am. chem. Soc.*, **69**, 1753 (1947)
(*13*) Sogami, M., Momotani, Y., and Inouye, Y., *J. Biochem.* **44**, 137 (1957)
(*14*) Sogami, M., *J. Biochem.*, **45**, 349 (1958)
Sogami, M., *J. Biochem.*, **45**, 367 (1958)
Sogami, M., *J., Biochem.*, in press
(*15*) Charlwood, P. A., *Biochem. J.*, **52**, 279 (1952)
(*16*) Charlwood, P. A., *Biochem. J.*, **51**, 113 (1952)
(*17*) Jirgenson, B., *Arch. Biochem.* and *Biophys.*, **59**, 420 (1955)
(*18*) Jirgenson, B., and Sirotzky, S., *Arch. Biochem. Biophys.*, **52**, 400 (1954)
(*19*) Luetscher, J. A., *J. Am. Chem. Soc.*, **51**, 2888 (1939)
(*20*) Luetscher, J. A., *J. Clin. Invest.*, **19**, 313 (1940)
(*21*) Saifer, A., and Corey, H., *J. Biol. Chem.*, **217**, 23 (1955)
(*22*) Saifer, A., and Corey, H., *Proc. Soc. Exptl. Biol. Med.*, **86**, 203 (1954)
(*23*) Alberty R. A., *J. Phys. Colloid Chem.*, **53**, 114 (1949)
(*24*) Hoch, H., and Morris, D. J. O. R., *Nature*, **156**, 234 (1945)
(*25*) Aoki, K., and Foster, J., *J. Am. Chem. Soc.*, **78**, 3538 (1956)
(*26*) Pheleps. R. A., and Cann, J. R., *J. Am. Chem. Soc.*, **78**, 3539 (1956)
(*27*) Larson, D. L., and Feinberg, R., *Science*, **120**, 426 (1954)
(*28*) Raacke, I. R., *Arch. Biochem. Biophys.*, **62**, 184 (1956)
(*29*) Keltz, A., and Mehl, J. W., *J. Am. Chem. Soc.*, **77**, 5764 (1955)
(*30*) Gordon, A. H., Gross, J., Oconor, E., and Pitt-River, R., *Nature*, **169**, 19 (1952)
(*31*) Larson, F. C., Deiss, W. P., and Albright, E. C., *Science*, **115**, 626 (1952)
(*32*) Deiss, W. P., Albright, E. C., and Larson, F. C., *J. Clin. Invest.*, **31**, 1000 (1952)
(*33*) Robbins, J., *Arch. Biochem. Biophys.*, **63**, 461 (1956)
(*34*) Slater, W. T., *Ann. N. Y. Acad. Sci.*, **50**, 358 (1949)
(*35*) Albright, E. C., Larson, F. C., and Deiss, E. P., *J. Clin. Invest.*, **34**, 44 (1955)
(*36*) Gleason., G. I., *J. Biol. Chem.*, **213**, 837 (1955)

Department of Physiology,
Faculty of Medicine,
Kyoto University, Kyoto

MASARU SOGAMI

(Received for publication, March 27, 1958)

昭和32年7月9日第三種郵便物認可
第45巻第6号昭和33年6月25日発行
(毎月1回25日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 6, June 25, 1958

Vol. 45 **No. 6**

CONTENTS

PAGE

SOGAMI, Masaru. Turbidometric titration of serum-protein. IV. Turbidometric titration of subfraction of bovine plasma albumin 367

TAKAHASHI, Kenji, TITANI, Koiti, FURUNO, Kyoko, ISHIKURA, Hisayuki and MINAKAMI, Shigeki. The structure of cytochrome c. III. The guanidination of cytochrome c—biologically active derivatives of cytochrome c 375

SAKURAI. Setsuji. Enzymatic preparation of optically active essential amino acids. IV. The preparation of L-leucine and L-valine. A comment on the transaminase systems of heart muscle 379

NADAI, Yukiaki. Colorimetric determination of urea with special reference of the arginase determination 387

SUGANO, Hiroshi. Studies on egg york proteins. III. Preparation and properties of two major lipoproteins of the egg pork, α- and β-lipovitellin 393

SUZUKI, Takeshi and ITO, Eiji. The effects of bromination on the ultraviolet absorption spectra of the purine and pyrimidine bases and the nucleosides 403

MATSUBARA, Hiroshi, and NISIMURA, Shigehiko. Crystalline bacterial proteinase. IV. Amino-terminal amino acid of crystalline bacterial proteinase 413

HAYAKAWA, Shohei, SABURI, Yasuo and TAMAKI, Keizo. Microbiological degradation of bile acids. XVIII. Separation and identification of the degradation products of cholic acid by *Streptomyces rubescens*.. 419

ANDO, Toshio, YAMASAKI, Makoto, ABUKUMAGAWA, Eiko, ISHII, Shin-ichi and NAGAI, Yutaka. Studies on protamines. III. N-Terminal residues of salmine and clupeine 429

ANDO, Toshio and HASHIMOTO, Chieko. Studies on protamines. IV. The basic proteins of the testis cell nuclei of adult and immature rainbow-trouts in breeding season 453

Letters to the Editors

SOGAMI, Masaru. Studies on the binding of labeled L-thyroxine with bovine plasma albumin subfractions 461